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Quantitation of β-lapachone and 3-hydroxy-β-lapachone in human plasma samples by reversed-phase high-performance liquid chromatography

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Abstract

β-Lapachone is an *o*-naphthoquinone found to have in vitro cytotoxicity in cancer cells, type I human immunodeficiency virus, and fungi. Analytical methods for evaluating β-lapachone in biological fluids using high-performance liquid chromatography (HPLC) have not been published. The reversed-phase HPLC method described in this report utilizes liquid extraction of a 0.5-ml plasma sample with average recoveries of 67±10.8% and 70±10.3% for β-lapachone and 3-hydroxy-β-lapachone, respectively. Sensitivity of the assay using ultraviolet (UV) detection at 256 nm is 15 ng ml^{-1} from a 100 μl injection. Plasma standards for β-lapachone and 3-hydroxy-β-lapachone are linear with no significant difference in slope between the compounds. The retention times of 2.7 min for 3-hydroxy-β-lapachone and 5.9 min for β-lapachone result in a clean separation permitting use of the same assay procedure without modification for both compounds. This assay offers the advantage that either β-lapachone or 3-hydroxy-β-lapachone can serve as the internal standard, depending on which compound is being analyzed.

Keywords: β-Lapachone; 3-Hydroxy-β-lapachone

1. Introduction

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol-[1,2-b]pyran-5,6-dione) can be prepared after extraction of lapachol, present in the lapacho tree (*Tabebuia avellanedae*) and lomatiol (obtained from the seeds of *Lomatia*) by partial synthesis or, alternatively, by means of total synthetic procedures [1–5]. Although the exact mechanism of action is unclear, studies have reported that β-lapachone inhibits topoisomerase I [6,7] and transcription of HIV-1 long terminal repeat (LTR) gene expression [8]. β-Lapachone has demonstrated in vitro chemotherapeutic potential in cancer, HIV-1 and fungal diseases [6–10].

The lipophilic nature of β-lapachone (see Fig. 1) is responsible for its low solubility in water (about 1 mg l^{-1}) but the compound is easily soluble in methanol, chloroform, dimethyl sulfoxide, or etha-

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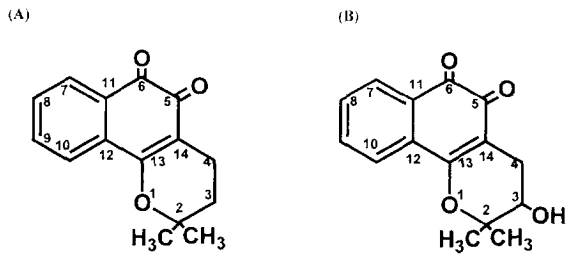

Fig. 1. Chemical structures of (A) β-lapachone and (B) 3-hydroxy-β-lapachone.

nol. The 3-hydroxy-β-lapachone compound was synthesized in an effort to improve the water solubility and its in vitro activity is not significantly different from β-lapachone. Stability of β-lapachone and other *o*-naphthoquinones is affected by exposure to light, air and changes in pH. β-lapachone can be reduced to a semiquinone radical upon photolysis in degassed solutions containing alcohols, amines and β-amino alcohols. The semiquinone radical is stable in anoxic environments but can regenerate back to β-lapachone in the presence of oxygen. Increasing the pH from 6.0 to 8.0 markedly enhances the susceptibility of the quinone moiety to oxidative processes [11,12]. Studies to evaluate the structure and activity of β-lapachone have used various methods such as NMR mass spectrophotometry, UV–Vis spectrophotometry and in vitro cell culture assays. However, these methods are not practical for routine quantitation of β-lapachone in biological fluids and tissues.

We report a reversed-phase HPLC method to determine the concentration of β-lapachone and 3-hydroxy-β-lapachone in human plasma. The results from this assay show that β-lapachone and 3-hydroxy-β-lapachone can be identified using HPLC with good separation of the compounds and a short run time less than 10 min.

2. Experimental

2.1. Chemicals

Organic solvents for mobile phase preparation and sample extraction included HPLC grade acetonitrile, chloroform, hexane, isopropanol and methanol (Fisher Scientific, Fair Lawn, NJ, USA). Triethylamine (Sigma, St. Louis, MO, USA) was used as an ion-pairing agent. The phosphate buffer solution was prepared with K_2HPO_4 (dibasic) and 85% phosphoric acid (Mallinckrodt, Paris, KY, USA). Trichloroacetic acid 12% (w/v) (Sigma) was used in a procedure for precipitation of plasma protein. Anhydrous sodium sulfate (Mallinckrodt) was used in the preparation of extracted samples. Filtering and vacuum-degassing of the mobile phase required the use of 0.45 μm nylon membranes (Alltech, Deerfield, IL, USA). β-Lapachone and 3-hydroxy-β-lapachone were synthesized and generously provided by Dr. Benjamin Frydman at the University of Wisconsin School of Pharmacy (Madison, WI, USA).

2.2. Mobile phase preparation

The mobile phase was a mixture (v/v) of 45% acetonitrile and 55% 0.01 *M* K_2HPO_4 containing 0.003% triethylamine. A stock solution of 0.01 *M* K_2HPO_4 was prepared by dissolving 1.74 g of anhydrous K_2HPO_4 in 1 liter of deionized water. The K_2HPO_4 solution used in mobile phase preparation was adjusted to a pH of 7.0 with 85% phosphoric acid after the addition of triethylamine. The mobile phase was filtered and vacuum-degassed using a 0.45-μm nylon membrane.

2.3. Sample preparation

Human plasma was centrifuged in a refrigerated centrifuge at 3600 *g* for 10 min to remove solids before preparation of the calibration standards. Stock 100 μg ml^{-1} solutions of β-lapachone and 3-hydroxy-β-lapachone were prepared in methanol and stored at −85 °C. The calibration standards consisted of a blank plasma sample in addition to samples containing either β-lapachone or 3-hydroxy-β-lapachone at concentrations of 15.6 ng ml^{-1}, 31.25 ng ml^{-1}, 62.5 ng ml^{-1}, 125 ng ml^{-1}, 250 ng ml^{-1}, 500 ng ml^{-1}, 1 μg ml^{-1} and 2 μg ml^{-1}. The 2 μg ml^{-1} standard was prepared by combining 0.1 ml of β-lapachone 100 μg ml^{-1} or 0.1 ml 3-hydroxy-β-lapachone 100 μg ml^{-1} in methanol with plasma in a volumetric flask to a total of 5 ml. A 1:1 serial dilution with plasma was used for preparation of all subsequent standards. Standards used to evaluate the recovery of β-lapachone and 3-hydroxy-β-lapachone

were prepared in methanol with the same method as the plasma standards.

The internal standard was prepared by diluting 0.1 ml of β-lapachone (100 μg ml^{-1}) or 0.1 ml 3-hydroxy-β-lapachone (100 μg ml^{-1}) in methanol with filtered, deionized water in a volumetric flask to 5 ml. To each sample, 0.1 ml (200 ng) of internal standard was added prior to extraction. The stock internal standard was stored at −85 °C.

2.4. Liquid extraction

A 0.5-ml plasma sample was placed in a silanized test tube followed by 5 ml of water-saturated chloroform (ether, hexane, and hexane with 10% isopropanol were also evaluated). The samples were placed on an orbital shaker at 2600 r.p.m. for 20 min and then centrifuged in a refrigerated centrifuge at 3600 *g* for 10 min. The organic phase was transferred to a clean silanized test tube and the remaining aqueous phase was re-extracted using the above procedure. The organic phase from both extractions was combined and 1 g of anhydrous sodium sulfate was added to the test tube and vortexed. The organic phase was transferred to a silanized, glass scintillation vial and evaporated under nitrogen. The dried sample was reconstituted in 0.25 ml of mobile phase and assayed.

2.5. Trichloroacetic acid extraction

In a 1.8-ml microcentrifuge tube, 0.5 ml of ice-cold trichloroacetic acid (12%, w/v) was added to a 0.5-ml plasma sample and thoroughly vortexed. The sample was centrifuged for 5 min and the clear supernatant was assayed.

2.6. Acetonitrile extraction

In a 1.8-ml microcentrifuge tube, 0.5 ml of ice-cold acetonitrile was added to a 0.5-ml plasma sample and vortexed. Then, 20 μl of a 10% (w/v) zinc sulfate solution was added to the microcentrifuge tube and vortexed again. The sample was centrifuged for 5 min and the clear supernatant was assayed.

2.7. HPLC conditions

A Beckman model 110B pump (Beckman, San Ramon, CA, USA) was set at a flow-rate of 2 ml min^{-1}. The mobile phase (1 liter) was recycled during assay runs and required fresh preparation after approximately 75 injections. Samples (100 μl) were injected using a Beckman model 506 autosampler with a 200 μl loop. A 2 cm×2 mm guard column (Upchurch, Oak Harbor, WA, USA) composed of 40 μm C_{18} packing preceded the analytical column. The analytical column was a NovaPak (100×8 mm I.D., 4 μm particles) phenyl Radial-Pak cartridge system (Waters, Milford, MA, USA).

A Beckman model 163 variable UV detector was set at a wavelength of 256 nm. Data were collected and analyzed using Beckman System Gold software, version 7.0.

3. Results

3.1. HPLC system

The maximum absorbance of β-lapachone in methanol was determined in the wavelength range of 200–600 nm using a Cary/Olis UV–Vis spectrophotometer (Olis, Jefferson, GA, USA). The spectrum showed three peaks at 256, 265 and 283 nm which agreed with literature reports [1,12,13]. The highest absorbance for both β-lapachone and 3-hydroxy-β-lapachone was observed at 256 nm. Although the HPLC system was initially developed with simultaneous absorbance and fluorescence detection, neither compound demonstrated fluorescence over a wide range of emission and excitation wavelengths. Therefore, UV detection proved to be the method of choice.

Different analytical columns and mobile phases were evaluated to determine optimal conditions. Mobile phases containing either acetonitrile (ACN) or methanol at concentrations of 20, 30 and 40% were evaluated with a C_{18} Radial-Pak cartridge (Waters). The retention time of β-lapachone with a mobile phase containing 40% methanol was 90 min and resulted in a broad, tailing peak. In comparison, 40% ACN in the mobile phase produced a sharp peak at 10 min.

Similar retention times giving poor separation of peaks were obtained on the C_{18} column for both 3-hydroxy-β-lapachone and β-lapachone. Therefore, a column with phenyl packing was evaluated with mobile phase containing various concentrations of ACN. The phenyl Radial-Pak column and mobile phase containing 45% ACN resulted in a clean separation of the compounds with retention times of 5.9 min and 2.7 min for β-lapachone and 3-hydroxy-β-lapachone, respectively (see Fig. 2).

3.2. Extraction method

Several extraction methods were evaluated to maximize the recovery of β-lapachone and 3-hydroxy-β-lapachone. Liquid extraction with water-saturated chloroform produced the best results with average recoveries of 67±10.8% and 70±10.3% for β-lapachone and 3-hydroxy-β-lapachone, respectively. The average recovery of β-lapachone and 3-hydroxy-β-lapachone was markedly lower with ether, hexane, or hexane with 10% isopropanol (see Table 1).

Precipitation of plasma protein using either ice-cold trichloroacetic acid (TCA) or ACN resulted in poor recovery of both compounds. Three 0.5-ml plasma samples containing 1 μg of β-lapachone or 3-hydroxy-β-lapachone were analyzed with each method. β-lapachone was not detectable after protein precipitation with TCA. Recovery of 3-hydroxy-β-lapachone ranged from 4–7% with an average recovery of 5±1.53%. The recovery of β-lapachone and 3-hydroxy-β-lapachone after protein precipitation with ACN ranged from 36–40% and 38–41% respectively, with an average recovery of 39±1.91% for both compounds.

3.3. Plasma calibration standards

Plasma calibration standards for β-lapachone and 3-hydroxy-β-lapachone were extracted with water-saturated chloroform as described above. Linear regression of the calibration data showed no significant differences in the slope or intercept of the compounds (see Table 2). Samples containing concentrations less than 15 ng ml^{-1} of β-lapachone were analyzed but the peaks were difficult to distinguish from the baseline noise (S/N ratio<3). Therefore, the limit of detection with a 0.5-ml plasma sample was 15 ng ml^{-1}.

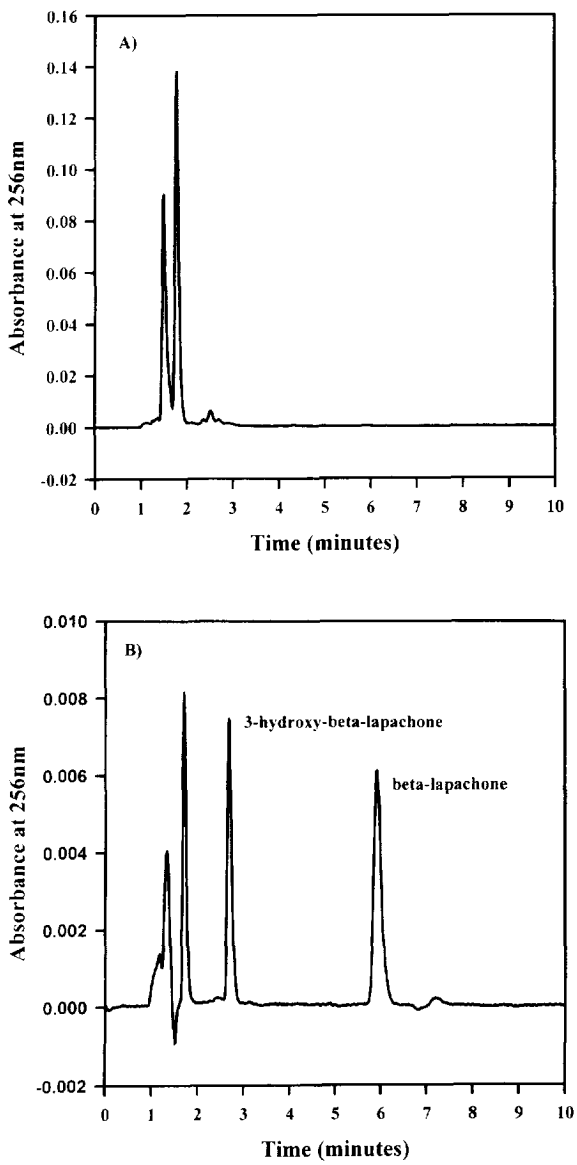

Fig. 2. Chromatograms of (A) blank plasma and (B) spiked plasma containing β-lapachone and 3-hydroxy-β-lapachone. The blank sample contained 0.5 ml of plasma. The spiked plasma sample (0.5 ml) contained 250 ng of β-lapachone with 200 ng of 3-hydroxy-β-lapachone as the internal standard. Samples were extracted with water-saturated chloroform. Retention times were 2.7 min for 3-hydroxy-β-lapachone and 5.9 min for β-lapachone.

Table 1
Comparison of recovery for β-lapachone and 3-hydroxy-β-lapachone with liquid extraction

Method	Average recovery (%)	Range (%)	S.D. (%)
β-Lapachone			
Chloroform	67	56–79	10.8
Diethyl ether	60	47–73	10.1
Hexane	57	51–65	6.81
Hexane–10% isopropanol	32	19–45	13.9
3-Hydroxy-β-lapachone			
Chloroform	70	57–80	10.3
Diethyl ether	68	60–74	5.06
Hexane	1.8	1.7–2	0.173
Hexane–10% isopropanol	23	16–37	9.70

The results represent recovery of the two drugs in a 0.5-ml plasma sample spiked with 1 μg of β-lapachone or 3-hydroxy-β-lapachone. Four samples were prepared and analyzed for each method.

4. Discussion

Reversed-phase HPLC resulted in good separation of β-lapachone and 3-hydroxy-β-lapachone. It was difficult to find an internal standard that had similar elution characteristics and could be detected at 256 nm. Commercially available 2-hydroxy-1,4-naphthoquinone was evaluated as an internal standard but eluted in the void volume even at low acetonitrile concentrations. Therefore, 3-hydroxy-β-lapachone proved to be a useful internal standard with a linear calibration similar to β-lapachone. Conversely, β-lapachone was used as the internal standard when analyzing 3-hydroxy-β-lapachone.

Both compounds have demonstrated nearly identical biological effectiveness on the inhibition of topoisomerase I in vitro. In our in vitro studies, 3-hydroxy-β-lapachone had slightly greater apoptosis in human promyelocytic leukemia (HL-60) cells and showed similar activity in various breast and prostate cancer cells compared to β-lapachone [14,15]. The advantage of using 3-hydroxy-β-lapachone is that the increased water solubility makes it easier to dissolve the compound in polar solvents that are less toxic to humans and animals. Although this assay is convenient because the method does not need to be modified for identification of either compound, there is no commercial source that supplies 3-hydroxy-β-lapachone. A successful search for commercially available compounds, which could be considered as suitable internal standard alternatives, would thus extend the applicability range of this assay.

Sensitivity of the assay was 15 ng ml^{-1} for β-lapachone which was near the limit of detection for the Beckman 163 variable UV detector. However, with a novel generation UV monitor, detection sensitivity would have been significantly improved over that obtained with the instrument available in our study. In addition, the *ortho*-benzoquinone moiety (i.e., at carbon atoms 5 and 6) would provide an ideal electrophor for its electrochemical detection in the reductive mode. For this reason, it can be

Table 2
Results for β-lapachone and 3-hydroxy-β-lapachone calibration standards extracted from plasma

Standard curve ($n=3$)	Slope (mean±S.E.)	Intercept (mean±S.E.)	Correlation coefficient
β-Lapachone	0.183±0.0044	0.0153±0.0182	0.998
3-Hydroxy-β-lapachone	0.181±0.0072	0.0207±0.0301	0.994

The results represent data obtained from the calibration curve of 0 ng ml^{-1} (blank), 15.6 ng ml^{-1}, 31.25 ng ml^{-1}, 62.5 ng ml^{-1}, 125 ng ml^{-1}, 250 ng ml^{-1}, 500 ng ml^{-1}, 1 μg ml^{-1}, and 2 μg ml^{-1} using 0.5-ml human plasma samples.

expected that optimisation of an electrochemical procedure will surely afford a dramatic decrease of either limits of detection or quantitation compared with the UV method. As a consequence, concentrations of the drug will still be measurable in that case, where only very low doses of β-lapachone are administered.

The liquid extraction method using water-saturated chloroform resulted in similar recovery for both β-lapachone and 3-hydroxy-β-lapachone. Sample preparation using solid-phase C_{18} extraction cartridges (Sep-Pak, Waters) was evaluated with β-lapachone but recovery results were unsatisfactory due to a rapid impairment of the chromatographic performance after several injections as a consequence of substantial deterioration of the analytical column. The estimated recovery of β-lapachone using Sep-Paks was 65%, which was similar to the results obtained with water-saturated chloroform. The advantage of using the liquid method was the elimination of variation due to factors such as flow-rate with Sep-Pak cartridges.

It is not clear what happened to β-lapachone after protein precipitation with TCA but the most likely explanation is that the acidic pH (2.0) of TCA caused a structural alteration of the molecule, which in turn, was also associated with corresponding changes in the UV absorption properties. Neither method of protein precipitation (TCA or ACN) resulted in recoveries for both compounds as high as liquid extraction with water-saturated chloroform. For this reason, studies evaluating the stability of β-lapachone and 3-hydroxy-β-lapachone at different pH values need to be conducted to determine optimal conditions for compound preparation and long term storage.

5. Conclusion

The reversed-phase HPLC method described in this report was designed to evaluate concentrations of β-lapachone and 3-hydroxy-β-lapachone in human plasma. Advantages of this method are that both compounds can be analyzed without modifying the assay and are eluted in less than 10 min. This method is currently being used to identify β-lapachone and 3-hydroxy-β-lapachone in murine samples and has demonstrated the same reproducibility as was reported here for the human plasma standards.

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