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Short communication

Rapid high-performance liquid chromatographic assay for atovaquone

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Abstract

A rapid high-performance liquid chromatography assay has been developed for the drug atovaquone, which is currently being used to treat *Pneumocystis carinii* pneumonia and *Toxoplasma gondii* encephalitis associated with the acquired immunodeficiency syndrome (AIDS). Protein is precipitated from plasma with acetonitrile–aqueous 1% acetic acid (85:15). The supernatant is assayed on a C_6 column using methanol–10 m*M* triethylamine in aqueous 0.2% trifluoroacetic acid (76:24) with detection at 254 nm. The working assay range was 0.5 to 50 μg/ml. Recovery was 97% and the between-day coefficients of variation were 2.1% at 50 μg/ml and 10.3% at 1 μg/ml. A number of drugs commonly used to treat AIDS and its complications did not interfere with the assay.

Keywords: Atovaquone

1. Introduction

Atovaquone (*trans*-2-[4-(4-chlorophenyl)-cyclohexyl]-3-hydroxy-1,4-naphthalenedione) is a potent inhibitor of the growth of various parasitic pathogens, including malaria species [1], *Pneumocystis carinii* [2] and *Toxoplasma gondii* [3]. Recently, atovaquone was approved by the US Food and Drug Administration for treatment of *P. carinii* pneumonia (PCP) in persons with the acquired immunodeficiency syndrome (AIDS) and intolerance to trimethoprim/sulfamethoxazole (TMP/SMX) [4]. It is also in clinical trials for treatment of *T. gondii* encephalitis in AIDS patients. Its advantages include oral activity, long half-life, and very low toxicity. Also, it appears to kill the parasites, while the alternative agents, TMP/SMX and pentamidine, only inhibit parasite growth [4,5].

Although its activity against PCP in clinical trials was less than that of TMP/SMX [6,7] or pentamidine [8], fewer patients had to discontinue treatment because of side effects. It has been suggested that failures were due to low plasma levels in some patients. Predicted effectiveness exceeded 95% for steady state plasma levels above 15 μg/ml [6]. Bioavailability varies markedly, is considerably lower in patients with AIDS than in asymptomatic volunteers and declines markedly when doses exceed 750 mg [4]. Therapeutic drug monitoring could improve clinical efficacy by identifying those patients who

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require an increased dose or a change to an alternate therapy due to inability to achieve adequate levels. The only assays published for atovaquone are a complex gas chromatographic method [9] and two HPLC methods requiring liquid–liquid extraction [10,11]. Of the HPLC methods, one does not use an internal standard [10]; the other has only minimal validation data [11]. We have developed a quick and simple direct protein precipitation HPLC method that should facilitate provision of atovaquone levels by clinical laboratories.

2. Experimental

Atovaquone and *trans*-2-hydroxy-3-(4-phenylcyclohexyl)-1,4-naphthalenedione (internal standard, I.S.) were provided by Burroughs-Wellcome (Research Triangle Park, NC, USA). Acetic acid, dimethylformamide and trifluoroacetic acid were reagent grade. All other reagents and solvents were HPLC grade. Calibrators and controls were prepared by supplementing outdated plasma from the American Red Cross (Farmington, CT, USA) with a stock standard containing 0.20 mg/ml atovaquone in 99:1 methanol–dimethylformamide. Calibrators were prepared at concentrations of 1, 2.5, 10, 20 and 50 μg/ml; blank plasma was used as a zero calibrator.

HPLC was carried out using a Spectroflow 400 pump (Kratos Analytical, Ramsey, NJ, USA) with a mixture of methanol–10 m*M* triethylamine in 0.2% (v/v) aqueous trifluoroacetic acid (pH 2) (76:24), pumped at 1.0 ml/min through a 250 × 4.6 mm Spherisorb C_6, 5 μm, column (Alltech, Deerfield, IL, USA) at 25°C. A Kratos Spectroflow 757 variable wavelength detector was used at 254 nm.

Prior to analysis, clinical specimens were heat-inactivated at 56°C for 1 h. Atovaquone has previously been shown to be stable to these conditions [10]. A 200 μl aliquot of plasma was mixed well with 50 μl of methanol–dimethylformamide (99:1) containing 100 μg/ml of I.S. Specimens were precipitated with 400 μl of acetonitrile–1.0% aqueous acetic acid (85:15), then centrifuged at 14 000 *g* for 3 min. Twenty μl of the supernatant was injected. Calibration used a linear least squares fit of atovaquone–I.S. peak-height ratios over the range 0–50 μg/ml.

Precision and analytical recovery were assessed by carrying out five replicate analyses daily for five days. Linearity of response was determined from a plot of the average peak-height ratios of four replicates of the seven calibrators. The limit of detection was set as three times the average baseline noise level in eight clinical specimens from untreated patients. Interference was assessed using these clinical specimens and 20 specimens from treated patients, as well as by direct injection of selected potentially interfering drugs (1–2 μg in 20 μl of protein precipitant).

3. Results and discussion

We have developed an HPLC method for the antiparasitic drug, atovaquone, that employs direct injection of the supernatant after precipitation and centrifugal removal of plasma proteins. Other published HPLC methods have required liquid–liquid extraction [10,11]. Atovaquone and the internal standard, *trans*-2-hydroxy - 3 - (4 - phenylcyclohexyl) - 1, 4 - naphthalenedione, were cleanly separated from plasma components (Fig. 1). Trifluoroacetic acid was used in the mobile phase to minimize on-column precipitation of any protein not removed in the precipitation step. Over 300 injections have been made without a degradation in column performance or significant increase in backpressure.

The method was linear to at least 50 μg/ml, as indicated by the relationship, observed concentration (μg/ml) = 49.99 ± 0.64 × relative dilution + 0.01 ± 0.26. The limit of detection was 0.5 μg/ml. At 1 μg/ml, recovery was 97.1%, the within-run coefficient of variation (C.V.) was 6.9% and the between-day C.V. was 10.3%. At 20 μg/ml, recovery was 97.1%, the within-run C.V. was 1.9% and the between-day C.V. was 2.1%.

Twenty specimens previously analyzed by the method of DeAngelis et al. [10] were reassayed

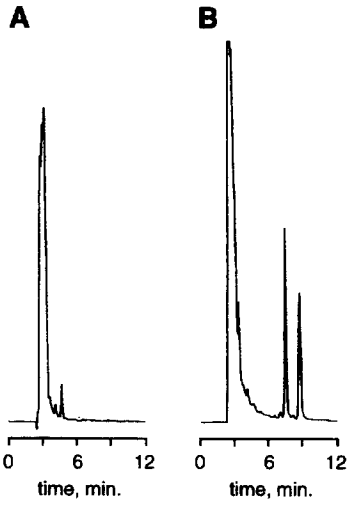

Fig. 1. (A) Chromatogram of plasma from an untreated patient. (B) Chromatogram of plasma from a patient with an atovaquone concentration of 19.9 μg/ml, spiked with internal standard. Retention times are: internal standard, 7.4 min; atovaquone, 8.8 min.

by the current method. Comparison of the values by a Deming regression gave the equation, $y = 0.937x + 0.90$, with an r value of 0.990. This improved to $y = 0.984x + 0.32$ ($r = 0.994$) after elimination of a single outlier.

The following drugs frequently used to treat AIDS and its complications were examined and shown to produce no interference: Clarithromycin (no peak seen), clindamycin (retention time, 4.2 min), dapsone, didanosine, fluconazole, folinic acid, foscarnet (no peak seen), ganciclovir, pentamidine (3.7 min), pyrimethamine (3.7 min), sulfadiazine, sulfamethoxazole, trimethoprim (3.3 min), trimetrexate, zalcitabine and zidovudine. Those substances for which a retention time is not given eluted at about 3 min in the complex of minimally retained substances.

In summary, we have developed a rapid, accurate HPLC method for atovaquone, and have shown it to be suitable for specimens from AIDS patients, who may be taking multiple other drugs simultaneously.

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References

[1] D.J. Hammond, J.R. Burchell and M. Pudney, Mol. Biochem. Parasitol., 14 (1985) 97.
[2] W.T. Hughes, V.L. Gray, W.E. Gutteridge, V.S. Latter and M. Pudney, Antimicrob. Agents Chemother., 34 (1990) 225.
[3] J. Huskinson-Mark, F.G. Araujo and J.S. Remington, J. Infect. Dis., 164 (1991) 101.
[4] W.T. Hughes, J. Acquired Immune Defic. Syndr. Hum. Retrovirol., 8 (1995) 247.
[5] F.G. Araujo, J. Huskinson and J.S. Remington, Antimicrob. Agents Chemother., 35 (1991) 293.
[6] W. Hughes, G. Leoung, F. Kramer, S.A. Bozzette, S. Safrin, P. Frame, N. Clumeck, H. Masur, D. Lancaster, C. Chan, J. Lavelle, J. Rosenstock, J. Falloon, J. Feinberg, S. LaFon, M. Rogers and F. Sattler, N. Engl. J. Med., 328 (1993) 1521.
[7] L.J. Epstein, Z. Mohsenifar, E.S. Daar, V. Yeh and R.D. Meyer, Am. J. Med. Sci., 308 (1994) 5.
[8] M.N. Dohn, W.G. Weinberg, R.A. Torres, S.E. Follansbee, P.T. Caldwell, J.D. Scott, J.C. Gathe, Jr., D.P. Haghighat, J.H. Sampson, J. Spotkov, S.C. Deresinski, R.D. Meyer, D.J. Lancaster and the Atovaquone Study Group, Ann. Int. Med., 121 (1994) 174.
[9] M.V. Doig and A.E. Jones, Methodol. Surv. Biochem. Anal., 20 (1990) 157.
[10] D.V. DeAngelis, J.D. Long, L.L. Kanics and J.L. Woolley, J. Chromatogr. B, 652 (1994) 211.
[11] P.E. Rolan, A.J. Mercer, B.C. Weatherley, T. Holdich, H. Meire, R.W. Peck, G. Ridout and J. Posner, Br. J. Clin. Pharmacol., 37 (1994) 13.