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Note

Determination of clofazimine in plasma by high-performance liquid chromatography

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Clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine; B663] is now an important secondary drug for use in patients infected with *Mycobacterium leprae* resistant to the primary drugs, dapsone and rifampin [1—3]. Early methods for the analysis of B663 in biological systems were based on the intense yellow-orange color of B663 in aqueous acid [4] or the production of a fluorescent derivative of B663 by reduction with titanous chloride [5]. Application of these methods by Levy [6] indicated that the practical limits of sensitivity for the colorimetric and fluorometric procedures were about 300 and 200 ng B663 per ml of plasma, respectively. Other workers [7] concluded that the two techniques were of equal sensitivities at about 200 ng/ml of plasma.

In this paper, we report the development of a method for measuring B663 in plasma with a limit sensitivity of about 10 ng/ml. This method involves extraction of B663 into organic solvents, separation of B663 from potential interfering materials by high-performance liquid chromatography (HPLC), and quantitation via the high absorbance of B663 at 285 nm. Following completion of our studies, Gidoh et al. [8] reported a similar HPLC method for B663.

EXPERIMENTAL

Materials

B663 was supplied by Dr. L. Levy (Hebrew University Hadassah Medical School, Jerusalem, Israel). Methanol, chloroform, and hexane were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); water was purified through a Super-Q water purification system (Millipore, Bedford, MA, U.S.A.). All solvents were filtered through a type HA filter (0.45 μm, Millipore); other chemicals were reagent grade.

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Screw cap glass culture tubes (50 ml) and 16 × 100 mm disposable culture tubes (Kimble, Toledo, OH, U.S.A.) were vapor-phase silylated [9], rinsed with methanol, and allowed to air-dry. The tubes were closed with foil-lined caps (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.). Injection vials for HPLC were disposable polypropylene microvials with polyurethane locking caps (Vanguard International, Neptune, NJ, U.S.A.).

Dr. R.R. Jacobson (Carville, LA, U.S.A.) kindly supplied us with heparinized plasma from his patients receiving B663 regimens therapeutically. We also used plasma from his patients receiving dapsone provided earlier [10] to test for interference by this drug and its metabolites and for recovery of B663 using the current method. The samples were stored and shipped frozen. We employed drug-free Sprague-Dawley rats (Simonsen Labs., Gilroy, CA, U.S.A.) as donors for heparinized rat plasma.

Extraction

B663 was extracted from 1.0-ml aliquots of rat or human plasma by addition of 1.0 ml of phosphate—citrate buffer, pH 6.0 (12.6 ml of 0.2 *M* Na_2HPO_4 and 7.4 ml of 0.1 *M* citric acid), and 14 ml of chloroform—methanol (4:1, v/v) in a 50-ml culture tube. The tube was closed with a foil-lined cap, and was shaken for 20 min at 80—100 strokes/min on a shaker (Eberbach, Ann Arbor, MI, U.S.A.). After centrifuging for 10 min at 400 *g*, the aqueous layer was aspirated off. Ten ml of the organic layer were transferred to a 16 × 100 mm test tube and evaporated to dryness under a gentle stream of high-purity nitrogen using a Meyer N-Evap (Organomation Assoc., Shrewsbury, MA, U.S.A.). The residue was reconstituted in 150 μl of mobile phase (0.0425 *M* phosphoric acid in 81% methanol) and 0.5 ml of hexane. Following centrifugation to separate the phases, the hexane layer was discarded and the mobile phase was transferred to an injection vial.

High-performance liquid chromatography

Samples were chromatographed using a Hewlett-Packard Model 1084B liquid chromatograph (Hewlett-Packard, Santa Clara, CA, U.S.A.) equipped with a variable-volume injector, automated sampling system, variable-wavelength detector, and automated fraction collector. B663 was separated from interfering plasma material using a reversed-phase Ultrasphere-octyl column (250 × 4.6 mm, 5-μm particle size; Altex, Berkeley, CA, U.S.A.) maintained at 40°C. Sample injection volume was 100 μl. The mobile phase for elution was 0.0425 *M* phosphoric acid in 81% methanol, pH 2.4, at a flow-rate of 1.5 ml/ min. Detection of B663 was accomplished by monitoring the column effluent at 285 nm. Plasma concentrations were quantitated by using integrated peak areas in comparison to standard quantities of B663 (in plasma) carried through the entire procedure.

Mass spectrometry

Fractions at the elution position of B663 were collected using the automatic fraction collector from extracts of plasma samples. B663 was extracted from the collected fractions with chloroform—methanol (4:1) and the organic phase was evaporated to dryness under a stream of nitrogen. The residue was sub-

jected to mass spectrometry using authentic B663 as the reference standard. Spectra were determined by direct introduction into an LKB 9000 mass spectrometer, using a 12-eV ionizing potential.

RESULTS

The elution profile of a plasma extract from a patient receiving B663 is shown in Fig. 1C. Fig. 1A shows the elution profile of an extract of control plasma and Fig. 1B the profile of a control plasma sample spiked with 332 ng/ml of B663. B663 exhibits a retention time of 9.6 min and is well resolved from extraneous UV-absorbing material. The antileprosy drugs, dapsone and ethionamide, eluted at the solvent front and rifampin was retained 3 min longer than B663.

To determine the recovery of B663 from plasma, pooled human plasma from patients receiving dapsone was used. The mean recovery of B663 from duplicate aliquots of plasma containing 33, 50, 330, and 830 ng/ml was 116% (S.D. = 10.4%; range was 97.3—130%). Duplicate determinations agreed within an average of 3.2% of their means. Fig. 2 shows a linear relationship between the peak areas of these samples and the quantity of B663 extracted ($r = 0.9981$, $P < 0.001$). The lower limit of sensitivity was estimated to be 10 ng/ml. Limited studies on the stability of B663 in frozen rat plasma showed that, at a concentration of 248 ng/ml, there was $< 2\%$ change in the level of B663 after nine days of storage. The mean recovery of 332 ng/ml of plasma on six different days was 105% (S.D. = 6.4%), thus demonstrating good reproducibility from day to day.

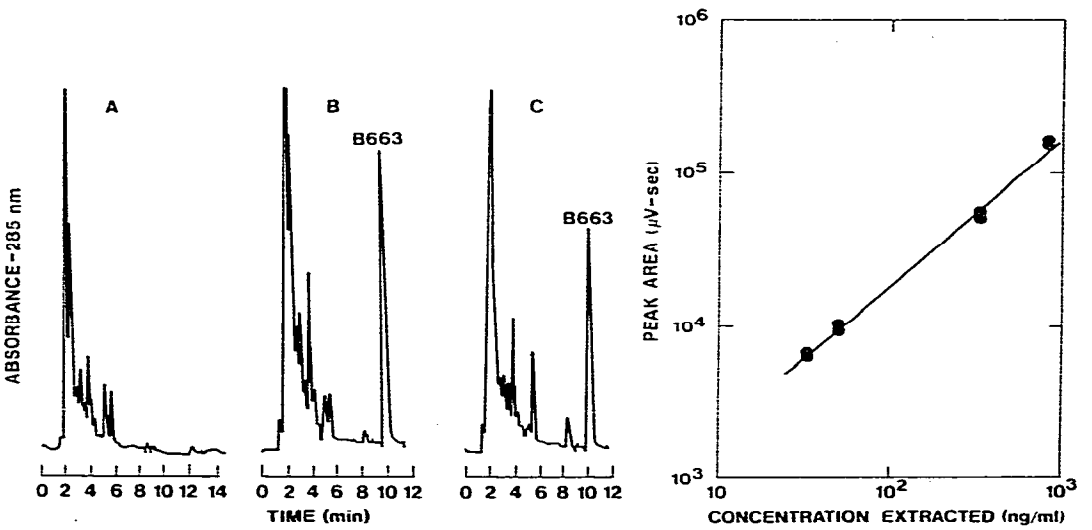

Fig. 1. Elution profiles of (A) B663-free plasma extract; (B) an extract of human plasma containing added B663; (C) an extract of a 2-h plasma sample from a patient receiving 100 mg B663 daily. The estimated level in the latter sample was 520 ng/ml of B663.

Fig. 2. Calibration plot of peak area at 285 nm versus concentration of B663 in plasma extracted.

Plasma levels of B663 in patients receiving B663 therapeutically are shown in Table I. All patients had received a dose of 100 mg B663 at zero time before the plasma samples were collected, regardless of their regimen. Patient 4 also received 250 mg ethionamide at zero time prior to sampling. The four patients (numbers 1, 2, 3, and 6) receiving 100 mg B663 daily exhibited variable times of maximum B663 levels from 2—8 h. These peak levels ranged from 550—1600 ng/ml in this group. Patient 4 receiving 250 mg ethionamide with 100 mg B663 daily was not different from the four receiving only B663 daily. Patient 5, receiving 100 mg B663 three times per week, exhibited the lowest levels of all patients; and patient 7, receiving 100 mg B663 three times per day, exhibited the highest levels at most time periods.

Because variable body weights of the patients could contribute to some of the differences found in the plasma levels of B663 of Table I, we have calculated the dose-adjusted levels shown in Table II wherein each plasma level was expressed in units of ng/ml divided by the mg B663 per kg of body weight of each patient. While these data can now be compared more rigorously, they do not eliminate the extreme variability in levels observed in the different patients. In all patients, a pattern of slow absorption and slow clearance from the body is indicated. At no time were levels found near the lower limit of our method. Also, no other peaks were seen on the chromatograms that indicate B663 metabolites. Finally, fractions collected at the retention time of authentic B663 from extracts of plasma exhibited mass spectra identical to that of authentic B663 (spectra are available from the authors on request).

DISCUSSION

Early methods for B663 depended upon absorption in visible light at about 540 nm [4,6,7] or conversion to a fluorescent derivative [5] but the practical limits of sensitivity of these techniques were 200—300 ng/ml of plasma [6]. In

TABLE I

LEVELS OF B663 IN PLASMA OF LEPROSY PATIENTS

Patient No.	Mean level (ng/ml) of B663 found after administration at				
	1 h	2 h	4 h	8 h	24 h
1*	350	460	610	590	410
2*	480	550	480	440	300
3*	1090	1030	1230	1600	1470
4**	— ***	630	660	680	490
5§	370	370	350	390	370
6*	570	630	640	700	510
7§§	1710	1880	2300	1270	1490

*Dose was 100 mg B663 daily.
**Dose was 100 mg B663 daily plus 250 mg ethionamide twice daily.
***No sample was available.
§Dose was 100 mg B663 three times per week.
§§Dose was 100 mg B663 three times per day.

TABLE II

DOSE-ADJUSTED LEVELS OF B663 IN PLASMA OF CARVILLE PATIENTS

Patient No.	Body weight (kg)	Hours after administration (ng/ml/mg B663/kg)				
		1	2	4	8	24
1*	52.7	184	245	323	309	215
2*	79.5	377	438	381	352	242
3*	63.6	696	657	783	1020	938
4**	92.7	—***	585	613	626	455
5§	70.9	265	261	245	278	260
6*	80.0	454	502	514	558	410
7§§	84.5	481	530	648	359	419

* Dose was 100 mg B663 daily.
** Dose was 100 mg B663 daily plus 250 mg ethionamide twice daily.
*** No sample was available.
§ Dose was 100 mg B663, three times per week.
§§ Dose was 100 mg B663, three times per day. For calculating dose-adjusted levels, a total single dose of 300 mg B663 was used.

our method, we have lowered the limit of sensitivity for measuring B663 to approximately 10 ng/ml of plasma using an original sample volume of 1 ml. Also, we have demonstrated no interference from plasma of patients receiving dapsone and separation of B663 from ethionamide and rifampin. Independently of us, Gidoh et al. [8] developed a similar HPLC method for B663. In their system, B663 exhibited an absorbance maximum at 282 nm (molar absorptivity = $8.8 \cdot 10^4$). In our mobile phase, we found the maximum for B663 and 285 nm (molar absorptivity = $8.4 \cdot 10^4$). Under these conditions, a second UV-absorbing peak for B663 was observed at 496 nm but the absorption was only one-half of that observed at 285 nm. We would expect that the method of Gidoh et al. [8] would be similarly applicable to analysis of plasma samples from patients receiving B663, although these authors reported only results of recoveries of B663 and other drugs added to pooled guinea pig serum. Their goal of development of a single method for measuring B663, dapsone, and rifampin has yet to be attained because the crucial tests of potential mutual interference by parent drug metabolites in human plasma in the assay of the other drugs have yet to be performed.

In the meantime, our current method has sufficient sensitivity and selectivity to measure B663 in patients receiving dapsone in combination with B663. Thus, in conjunction with methods we have already published for the primary antileprosy drugs, dapsone [11] and rifampin [12], and the newer secondary drugs, ethionamide and prothionamide [13], it is now possible to monitor antileprosy drug therapy of patients receiving combinations of these drugs. Recently, we have shown that saliva can be employed instead of plasma for monitoring dapsone therapy [14].

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