ELSEVIER

Journal of Chromatography B, 655 (1994) 89–96

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

Simultaneous determination of primaquine and carboxyprimaquine in plasma using high-performance liquid chromatography with electrochemical detection

Robert A. Dean[a,b,*], Willis Ochieng[c], John Black[d], Sherry F. Queener[e], Marilyn S. Bartlett[a], Natividad G. Dumaual[a]

[a] *Department of Pathology and Laboratory Medicine, School of Medicine, Indiana University, Indianapolis, IN, USA*
[b] *Department of Biochemistry and Molecular Biology, School of Medicine, Indiana University, Indianapolis, IN, USA*
[c] *Department of Forensic Medicine and Toxicology, Moi University Medical School, Eldoret, Kenya*
[d] *Department of Medicine, School of Medicine, Indiana University, Indianapolis, IN, USA*
[e] *Department of Pharmacology and Toxicology, School of Medicine, Indiana University, Indianapolis, Indiana, USA*

(First received October 19th, 1993; revised manuscript received January 31st, 1994)

Abstract

A selective and sensitive high-performance liquid chromatographic method with electrochemical detection is described for the simultaneous quantitation of primaquine and carboxyprimaquine, its primary metabolite, in plasma. After addition of internal standard, plasma was deproteinized by addition of acetonitrile. Nitrogen-dried supernatants, resuspended in mobile phase were analyzed on a C_8 reversed-phase column. Limits of detection for primaquine and carboxyprimaquine were 2 and 5 ng/ml with quantitation limits of 5 and 20 ng/ml, respectively. None of 47 tested antimicrobial agents interfered. In contrast to previously reported methods, the assay sensitivity and specificity are sufficient to permit quantitation of primaquine in plasma for pharmacokinetics following low dose (30 mg, base) oral administration of primaquine, typically used in the treatment of malaria and *Pneumocystis carinii* pneumonia.

1. Introduction

Primaquine is the drug of choice for radical cure of *Plasmodium vivax* malaria [1], and has been used to treat chloroquine-resistant malaria [2]. It also has proven an effective prophylactic against *Plasmodium falciparum* and *Plasmodium vivax* [3]. More recently, primaquine when combined with clindamycin was found to be effective in both the treatment and prevention of pneumonia due to *Pneumocystis carinii* in immunosuppressed animal models [4-6]. This combination, which may be less toxic than trimethoprim–sulfamethoxazole or pentamidine, has been utilized successfully as primary therapy in individuals suffering from acquired immune deficiency syndrome (AIDS) and *Pneumocystis* pneumonia of mild to moderate severity [7–9]. Primaquine–clindamycin therapy also has proven effective as salvage therapy for *Pneumocystis* pneumonia unresponsive or intolerant to conventional drug regimens [9,10].

* Corresponding author.

0378-4347/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(94)00087-L

The effectiveness of the primaquine–clindamycin combination against *Pneumocystis carinii* led us to investigate analytical methods for therapeutic drug monitoring and pharmacokinetic investigations following low-dose primaquine administration. Gas chromatographic (GC) methods for primaquine analysis utilize electron-capture detection (ECD) [11], nitrogen-selective detection (NSD) [12] or mass spectrometry (MS) [13]. Despite complicated sample preparation including drug derivatization, the sensitivity of these methods is insufficient for plasma analysis of primaquine at a dose typical for treatment of malaria or *Pneumocystis carinii* pneumonia. High-performance liquid chromatographic (HPLC) methods utilizing ultraviolet (UV) detection [3,12,14] or electrochemical detection (ED) [15] also have been reported. HPLC-UV methods exhibit inadequate sensitivity for plasma analysis following low-dose primaquine administration. The HPLC-ED method, while exhibiting excellent sensitivity for primaquine and the metabolite carboxyprimaquine, provides inadequate specificity for the study of human subjects receiving multiple drug therapy. Accordingly, we have developed an HPLC-ED assay for primaquine and carboxyprimaquine with sensitivity and specificity adequate for clinical and experimental use following low-dose (30 mg, base) primaquine administration in combination with other chemotherapeutic agents used in the management of patients suffering from AIDS.

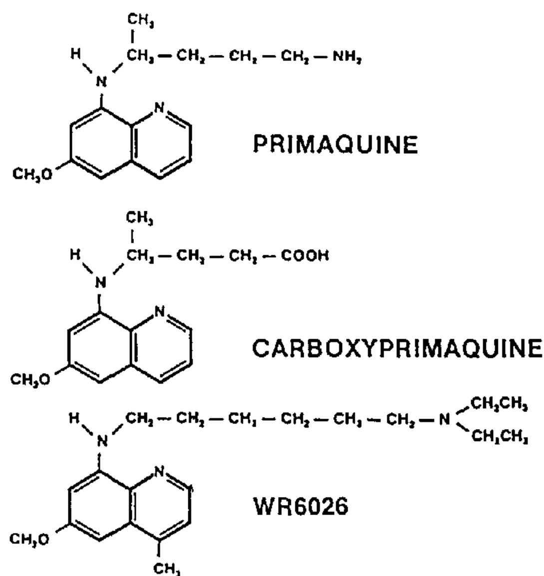

Fig. 1. Molecular structures of primaquine, carboxyprimaquine and the internal standard, WR6026.

2. Experimental

2.1. Chemicals and reagents

Primaquine (PQ), 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline (Fig. 1) was purchased from Aldrich (Milwaukee, WI, USA). Carboxyprimaquine (CPQ), 8-(3-carboxy-1-methyl-propylamino)-6-methoxyquinoline (Fig. 1) was provided by Dr. James D. McChesney, University of Mississippi [16]. WR6026 (Fig. 1), WR238605 and WR242511 were provided by the Walter Reed Army Institute of Research (WRAIR) (Washington, DC, USA). All other drugs were obtained from commercial manufacturers. HPLC-grade methanol and acetonitrile were from Baxter Scientific Products (McGaw Park, IL, USA). Decanesulfonic acid was from Sigma (St. Louis, MO, USA). Plasmanate, a plasma protein fraction prepared from pooled human plasma, was obtained from Miles (Etobiocoke, Ont., Canada).

2.2. Preparation of standards and controls

Stock standards of primaquine, carboxyprimaquine and WR6026 were prepared in methanol at 1 mg/ml as free base. Working standards and controls were prepared in plasmanate from separate stock standards. Stock standards, working standards and controls were stored at −20°C protected from light. WR6026 (internal standard; 10 μg/ml) was prepared in methanol.

2.3. Patient samples

The participation of human subjects in this study was reviewed and approved by the Institu-

tional Review Board, Indiana University School of Medicine, and was consistent with the Helsinki Declaration of 1975, as revised in 1983. Plasma samples were obtained at 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 12, 16 and 24 h following oral administration of 30 mg primaquine as two 26.3 mg primaquine phosphate tablets (Sterling Winthrop). Plasma samples were stored in the dark at −20°C until assayed.

2.4. Sample extraction

Five hundred μl of patient, standard and control samples were combined with 100 μl of internal standard in a polypropylene tube. Following addition of 750 μl acetonitrile, each sample was sealed, mixed and placed in the dark at 4°C for 60 min. Supernatants, prepared by centrifugation at 9500 *g* for 5 min, were transferred to a polypropylene tube and dried under a stream of nitrogen for approximately 45–60 min. The concentrates were reconstituted with 100 μl mobile phase and centrifuged at 9500 *g* for 5 min. The supernatants were transferred to a polypropylene sampling vial and a 10-μl aliquot was injected onto the HPLC system.

2.5. Apparatus and chromatographic conditions

Analyses were performed on an HP 1090 liquid chromatograph with an HP 3396A integrator from Hewlett-Packard (Eventual, PA, USA). The column, an Ultrasphere C_8 column (15 cm × 4.6 mm I.D., 5 μm) from Beckman (San Ramon, CA, USA), was maintained at 35°C. Electrochemical (ED) detection was achieved using a CC-4 electrochemical cell and an LC-4B controller from Bioanalytical Systems (West Lafayette, IN, USA). The ED detector was operated at + 0.82 V. The mobile phase was 7.0 m*M* monochloroacetic acid, 0.5 m*M* 1-decanesulfonic acid–acetonitrile–methanol (56:24:20, v/v). The flow-rate was 1.4 ml/min. Equilibration of the HPLC-ED system required 30–45 min. The potential of the ED detector was optimized with the mobile phase. Passivation of the electrode necessitated cleaning following every 100 injections.

2.6. Assay validation

Absolute recovery (extraction efficiency) from plasmanate was determined by quadruplicate analysis of high (1000 ng/ml) and low (100 ng/ml) concentration controls. Peak heights for high and low controls were compared to peak heights for standards prepared to equal 100% recovery. Within- and between-run precision was determined by repeated analysis of high and low concentration controls in a single run and in repeated separate day runs, respectively. Calibration curves were constructed by plotting the ratios of peak heights for primaquine and carboxyprimaquine to the peak height for the internal standard *vs.* concentration. The limit of detection was defined as a discernable signal at least 3-fold greater than that of the baseline noise. The limit of quantification was defined as the concentration at which quadruplicate within-run analysis of standards had a coefficient of variation less than or equal to 20%. Specificity was examined by assessing the potential interference produced by 47 drugs prepared at a concentration of 100 μg/ml.

2.7. Pharmacokinetics

To demonstrate the utility of this analytical method, data obtained by analysis of human plasma following oral administration of 30 mg primaquine were fitted to an open, two-compartment, pharmacokinetic model assuming first order drug absorption and elimination and providing for lag time in drug absorption. The program utilized, PCNONLIN (Version 3.0), employs iterative, nonlinear least-squares regression. It was obtained from SCI Software (Lexington, KY, USA).

3. Results and discussion

The HPLC-ED assay described in the present study combines the optimal features of previous

methods for primaquine and carboxyprimaquine. This approach provides advantages over previously reported methods. The absolute recovery (extraction efficiency) of primaquine and carboxyprimaquine by acetonitrile extraction was previously reported to be 98% [3]. We were unable to achieve these very high absolute recoveries and found extraction efficiencies for primaquine and carboxyprimaquine in the range 73–80% and 87–95%, respectively. Nevertheless, the extraction efficiencies using acetonitrile were consistently higher than those achieved with other solvents, and the analytical recoveries (corrected for recovery of internal standard) for primaquine and carboxyprimaquine were consistently greater than 98%. Moreover, acetonitrile extraction provided easy sample preparation with simultaneous deproteinization. In addition, acetonitrile extraction using polypropylene materials avoided the need to silanize the glassware as required by other methods [11,14].

The calibration curve for primaquine was linear from 5 to 500 ng/ml (Fig. 2). The slope and y-intercept ± standard error were 1.6 ± 0.02 and −5.8 ± 16.3, respectively. The coefficient of determination (r^2) was 0.997. The calibration curve for carboxyprimaquine was linear from 20 to 1000 ng/ml (Fig. 3). The slope and y-intercept ± standard error were 5.7 ± 0.02 and −72 ± 160, respectively. The coefficient of determination (r^2) was 0.997. The within- and between-run assay precision and analytical recovery for low and high concentration primaquine and carboxyprimaquine controls are shown in Table 1. Use of the 8-aminoquinoline WR6026 as an internal standard served to maintain assay precision and analytical recovery over a broad concentration range.

The limits of detection (sensitivity) for primaquine and carboxyprimaquine were 2 and 5 ng/ml, respectively. The limits of quantification for primaquine and carboxyprimaquine were 5 and 20 ng/ml, respectively. The sensitivity of this assay was greater than that achieved by gas chromatography with electron-capture [11], nitrogen-selective ionization [12], or mass spec-

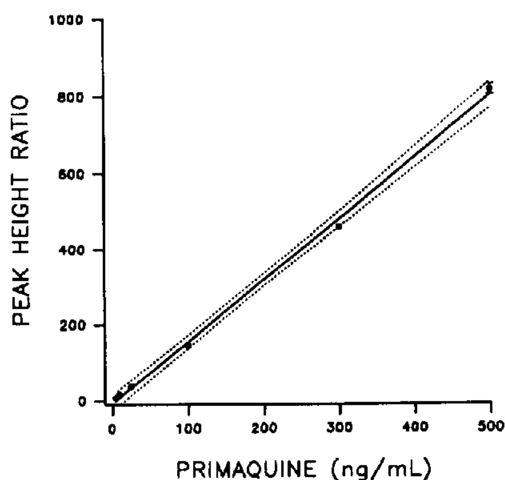

Fig. 2. Assay linearity for primaquine. The coefficient of determination (r^2) over the dynamic range (5–500 ng/ml) for the primaquine calibration curve was 0.997. The slope and y-intercept are listed under Results. The points represent the mean ± the standard error ($n = 5$). No vertical bars around the mean indicates that the standard error is within the limits of the mean symbol. The solid and dotted lines represent the regression line and 95% confidence interval, respectively.

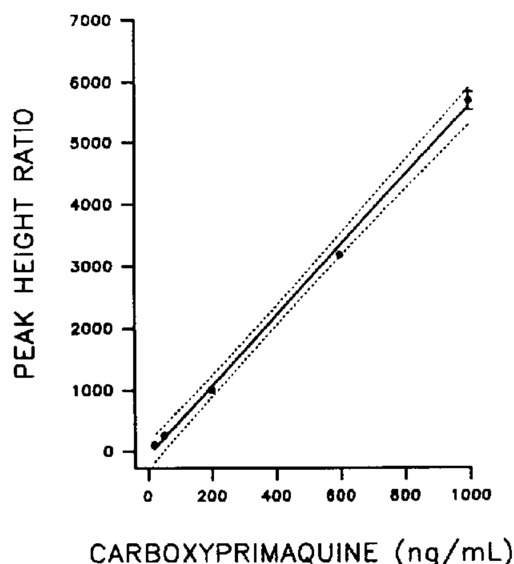

Fig. 3. Assay linearity for carboxyprimaquine. The coefficient of determination (r^2) over the dynamic range (20–1000 ng/ml) for the carboxyprimaquine calibration curve was 0.997. The slope and y-intercept are listed under Results. The points represent the mean ± the standard error ($n = 5$). No vertical bars around the mean indicates that the standard error is within the limits of the mean symbol. The solid and dotted lines represent the regression line and 95% confidence interval, respectively.

Table 1
Within- and between-run analytical precision and recovery

Compound	Target concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Analytical recovery (%)
Within-run				
Primaquine	50	50 ± 2	4.0	100
	400	409 ± 12	2.9	102
Carboxyprimaquine	100	98 ± 2	2.0	98
	800	794 ± 19	2.4	99
Between-run				
Primaquine	50	53 ± 4	7.5	106
	400	399 ± 14	3.5	100
Carboxyprimaquine	100	109 ± 14	12.8	109
	600	805 ± 70	8.7	101

The mean and standard deviation (S.D.) are based upon replicate analysis of each control ($n = 10$). Analytical recovery is expressed as a percentage of the determined mean concentration relative to the target concentration. Precision is expressed as the coefficient of variation percent (C.V.%).

trometric [13] detection. The sensitivity also was greater than that obtained by HPLC with UV detection [3,12,14] and comparable to that achieved by dual-electrode electrochemical detection [15]. This level of sensitivity was sufficient for pharmacokinetic analysis of plasma following single, low-dose (30 mg) oral administration of primaquine (see below).

The chromatographic conditions for this assay produced clear resolution of primaquine, carboxyprimaquine and the internal standard (Fig. 4). Although the retention time (t_R) for peaks corresponding to primaquine, carboxyprimaquine and the internal standard were stable within a given run, modest shifts in retention times were noted from run to run. Despite these shifts, the retention times of primaquine (PQ) and carboxyprimaquine (CPQ) expressed as a ratio relative to the internal standard (I.S.) were highly reproducible ($t_{R(PQ)}/t_{R(I.S.)}$ and $t_{R(CPQ)}/t_{R(I.S.)}$). Reversed-phase HPLC on a C_8 column and use of 1-decanesulfonic acid, an ion-pairing reagent, in the mobile phase reduced interferences from unidentified plasma constituents, which we observed using a previously reported HPLC-ED method [15]. Example chromatograms illustrate the resolution of primaquine, carboxyprimaquine and the internal standard

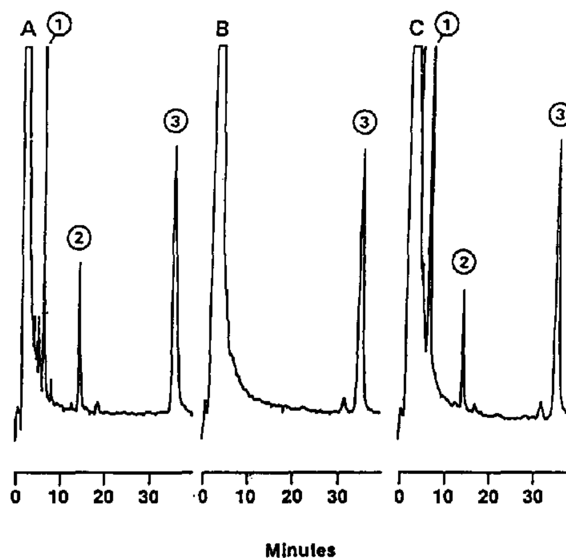

Fig. 4. Chromatographic separation of primaquine and carboxyprimaquine. Carboxyprimaquine (1), primaquine (2) and the internal standard, WR6026 (3) were resolved using the HPLC system described under Experimental. Chromatogram A is a plasmanate standard containing 200 ng/ml carboxyprimaquine and 100 ng/ml primaquine. Chromatogram B is human plasma obtained prior to primaquine administration. Chromatogram C is plasma obtained from the same individual 1.5 h following oral administration of 30 mg primaquine.

from plasma constituents (Fig. 4). Potential interference by 47 chemotherapeutic agents (parent compounds) also was examined and none was identified (Table 2). The demonstrated specificity of this assay is important as it permits reliable quantitation of primaquine and carboxyprimaquine in patients receiving multiple drug therapy.

The utility of this assay is demonstrated by pharmacokinetic analysis of plasma obtained from an AIDS patient following a single oral 30-mg dose of primaquine (Fig. 5). This is a typical oral dose of primaquine for treatment of *Pneumocystis carinii* and the lowest dose used to demonstrate the adequacy of a chromatographic method for pharmacokinetics studies. The plasma concentration *vs.* time profile for absorption, distribution and elimination of primaquine in man resembles that previously demonstrated following single, higher oral doses [3,14,15]. Moreover, absorption lag time (0.5 h), the absorption halflife (0.15 h), and the terminal elimination halflife (2.9 h) are consistent with that previously reported following administration of a single, higher dose of primaquine [3,17]. The area under the plasma concentration *vs.* time curve defined from zero to infinity (940 ng ml^{-1} h) was proportionally less than that previously noted in individuals receiving higher doses of primaquine [17]. This latter observation is consistent with the high correlation between the area under the curve and the primaquine dose [18]. The maximum plasma concentration (149 ng/ml) observed in the present study was essentially identical to that reported for an individual receiving primaquine as a single oral 45-mg (free

Table 2
Assay specificity

Drug	*k'*	Drug	*k'*
Amikacin		Erythromycin	
Amoxacillin		Gentamicin	
Ampicillin		Imipenem	
Azlocillin		Kanamycin	
Aztreonam		Lincomycin	
Carbenicillin	8.8	Methicillin	
Carboxyprimaquine	2.9	Minocycline	5.9
Cefaclor		Moxalactam	5.9
Cefamandole	9.1	Nafcillin	
Cefazolin		Neomycin	
Cefonicid		Netilmicin	
Cefoperazone		Oxacillin	
Cefotetan		Penicillin	
Cefoxitin		Piperacillin	
Ceftazidime		Primaquine	7.5
Ceftizoxime		Rifampin	
Ceftriaxone		Streptomycin	
Cephalothin		Sulfamethoxazole	
Cephalexin		Tetracycline	
Chloramphenicol		Ticarcillin	
Chloroquine		Trimethoprim	
Ciprofloxacin		WR6026 (ISTD)	20.7
Clindamycin		WR238605	>30
Cloxacillin		WR242511	>30
Dicloxacillin	12.9	Zirovudine	

The drugs listed were prepared at a concentration of 100 μg/ml and found not to interfere with the chromatographic analysis of primaquine and carboxyprimaquine. The capacity factor (*k'*) was determined for the drugs which were detected. The remaining drugs were not detected.

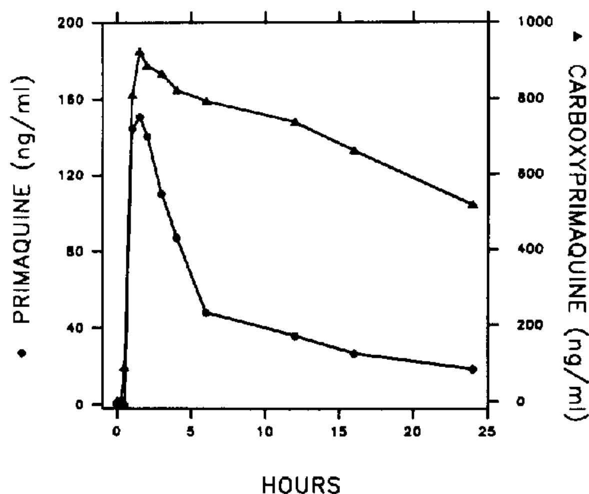

Fig. 5. Plasma concentration of primaquine and carboxyprimaquine *versus* time. The time course for primaquine and carboxyprimaquine plasma levels in an AIDS patient was determined following oral administration of 30 mg primaquine.

base) dose [14]. This relatively high maximum plasma concentration is likely due to more rapid absorption as reflected in the short time to maximum plasma concentration (1.2 h) when compared to the previously reported case (time to reach maximum concentration = 2.6 h) [14]. Finally, rapid metabolism of primaquine to carboxyprimaquine was confirmed (Fig. 5) [12].

Although initially developed for analysis of primaquine and carboxyprimaquine in plasma, this method also can be used for quantitation of these compounds in other biological specimens. In particular, we have used this method to measure primaquine and carboxyprimaquine in homogenates of rodent tissues including lung, liver and brain. When applied to these other tissues however, it is critical that calibrators be prepared in the same tissues which are drug free and acetonitrile-treated. Finally, by using primaquine as an internal standard, this assay also can be used to measure WR6026, another 8-aminoquinoline with activity against *Pneumocystis carinii* in rodent models [6]. WR6026 is currently being investigated in human studies for treatment of *Pneumocystis carinii* pneumonia and diseases caused by *Leishmania donovoni* [19].

The combination of primaquine and clindamycin represents a promising therapy for treatment of *Pneumocystis carinii* pneumonia. Mucosal disease in the gastrointestinal tract, however, could potentially decrease the absorption and, thus, the bioavailability of primaquine administered by the oral route. Accordingly, therapeutic monitoring of plasma primaquine may prove clinically important, identifying patients that might not respond to this therapy. This concern is especially relevant to patients with severe *Pneumocystis* pneumonia, unresponsive or intolerant to conventional therapies. The present method provides analytical sensitivity sufficient for quantitation of primaquine and carboxyprimaquine in plasma following low dose primaquine administration, as used in treatment and prophylaxis for *Pneumocystis* pneumonia. Moreover, the specificity of this method permits therapeutic drug monitoring in AIDS patients requiring administration of multiple pharmaceutical agents.

4. Acknowledgements

The advice and suggestions offered by Dr James W. Smith and Dr. James D. McChessney were greatly appreciated. This work was supported in part by NIH Contract No. N01-AI-72647.

5. References

[1] J.H. Edgcomb, J. Arnold, E.H. Yount, A.S. Alving, L. Eichelberger, G.M. Jeffery, D. Eyles and M.D. Young, *J. Natl. Malaria Soc.*, 9 (1950) 285.
[2] R.E. Strube, *J. Trop. Med. Hyg.*, 78 (1975) 171.
[3] G.W. Parkhurst, M.V. Nora, R.W. Thomas and P.E. Carson, *J. Pharm. Sci.*, 73 (1984) 1329.
[4] S.F. Queener, M.S. Bartlett, J.D. Richardson, M.A. Durkin and J.W. Smith, *Antimicrob. Agents Chemother.* 32 (1988) 807.
[5] J.W. Smith, *Eur. J. Clin. Microbiol. Infect. Dis.*, 10 (1991) 201.
[6] S.F. Queener, R.A. Dean, M.S. Bartlett, W.K. Mihous, J.D. Berman, W.Y. Ellis and J.W. Smith, *J. Infect. Dis.*, 165 (1992) 764.

[7] E. Toma, S. Fournier, M. Poisson, R. Morisset, D. Phaneuf and C. Vega, *Lancet*, (1989) 1046.
[8] J.R. Black, J. Feinberg, R. Murphy, R.J. Fass, J. Carey and F.R. Sattler, *Eur. J. Clin. Microbiol.*, 10 (1991) 204.
[9] O. Kay and R.E. DuBois, *South Med. J.*, 83 (1990) 403.
[10] G.A. Noskin, R.L. Murphy, J.R. Black and J.P. Phair, *Clin. Infect. Dis.*, 14 (1992) 183.
[11] T.G. Rajagopalan, B. Anjaneyulu, V.D. Shanbag and R.S. Grewal, *J. Chromatogr.*, 224 (1981) 265.
[12] J.K. Baker, J.D. McChesney, C.D. Hufford and A.M. Clark, *J. Chromatogr.*, 230 (1982) 69.
[13] J. Greaves, D.A. Price-Evans, H.M. Gilles and J.D. Baty, *Biomed. Mass Spectrom.*, 6 (1979) 109.
[14] S.A. Ward, G. Edwards, M.L.E. Orme and A.M. Breckenridge, *J. Chromatogr.*, 305 (1984) 239.
[15] M.V. Nora, G.W. Parkhurst, R.T. Thomas and P.E. Carson, *J. Chromatogr.*, 307 (1984) 451.
[16] J.D. McChesney and S. Sarangan, *Pharm. Res.*, 2 (1984) 96.
[17] K.A. Fletcher, D.A. Price Evans, H.M. Gilles, J. Greaves, D. Bunnag and T. Harinasuta, *Bull. WHO*, 59 (1981) 407.
[18] G.W. Milhaly, S.A. Ward, G. Edwards, D.D. Nicholl, M. L'E Orme and A.M. Breckenridge, *Br. J. Clin. Pharmacol.*, 19 (1985) 745.
[19] B. Petty, J. Black, C. Hendrix, Y. Bassiakos, J. Feinberg, R. Hafner, G. Simpson, W. Rickard, L. Lewis, D. Pattison and P. Lietman, *IX International Conference on AIDS*, Berlin, Abstract (June 1993).