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Simultaneous determination of chloroquine and quinine in human biological fluids by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method with fluorescence detection is described for the simultaneous measurement of quinine, chloroquine and mono- and bidesethylchloroquine in human plasma, erythrocytes and urine. After a liquid–solid extraction on a Bond Elut C_8 cartridge, the compounds are separated on an Inertsil silica column by gradient elution; the mobile phase is a mixture of acetonitrile and methanol–25% ammonia solution (92.7:7.5, v/v). The eluent was monitored with a fluorescence detector (excitation wavelength 325 nm and emission wavelength 375 nm). The limit of detection was *ca.* 5 ng/ml for chloroquine and *ca.* 23 ng/ml for quinine. No chromatographic interferences could be detected from endogenous compounds or other antimalarial drugs. The method is accurate with inter- and intra-assay coefficients of variation lower than 7%. Hydroxychloroquine is used as an internal standard because of its structural similarity to chloroquine. The procedure requires 30 min and can be used for therapeutic drug monitoring.

INTRODUCTION

Quinine (Q), an alkaloid extracted from the bark of the cinchona tree, has been used as an antimalarial drug since 1663. Chloroquine (CQ), a 4 aminoquinoline derivative, is among the most commonly used compounds for the chemoprophylaxis and treatment of malaria; however, as chloroquine-resistant *Plasmodium falciparum* is now widespread, quinine has received renewed interest and remains the drug of choice for treating acute attacks and complicated malaria in areas where parasite-resistant strains are most dominant. Quinine is a rapid-acting blood schizontical drug but this cinchona alkaloid has a narrow therapeutic index with adverse reactions usually called "cinchonism"; hence drug concentration monitoring in biological fluids is necessary for clinical management. The determination of chloroquine blood levels is a predictive tool for asserting patient chemoprophylaxis compliance, establishing whether therapeutic concentrations have been obtained or for suggesting the presence of a resistant strain of the parasite.

The analytical method of choice for measuring the antimalarial drugs administered either as a monotherapy or in associations is high-performance liquid chromatography (HPLC). Several

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methods have been reported for the determination of CQ concentration; these methods include the use of normal- or reversed-phase columns after liquid–liquid extraction of the drugs and either ultraviolet (UV) or fluorescence detection [1–10]. The latter is better because of its greater sensitivity. In contrast to CQ, fewer methods for the determination of Q in biological fluids have been reported; either normal- or reversed-phase chromatographic separations have been described coupled with fluorescence [11–16] or UV detection.

The purpose of this study was to develop a simple and selective HPLC method for the simultaneous measurement of Q, CQ and its two major metabolites, monodesethylchloroquine (MDCQ) and bidesethylchloroquine (BDCQ), in various biological fluids. The simultaneous detection of the two drugs reduces substantially the overall cost of analysis and the rapid liquid–solid extraction make the method suitable for routine monitoring of Q and CQ levels in patients.

EXPERIMENTALS

Chemicals and reagents

All chemicals were of analytical-reagent grade unless indicated otherwise. Ammonium formate was purchased from Sigma (St. Louis, MO, USA), 25% ammonia solution and HPLC-grade methanol from Merck (Darmstadt, Germany) and acetonitrile (special reagent for HPLC) from Carlo Erba (Rueil-Malmaison, France). Bond Elut C_8 cartridges were obtained from Prolabo (Paris, France). Chloroquine sulphate, monodesethylchloroquine base, bidesethylchloroquine base and hydroxychloroquine sulphate (internal standard, I.S.) were kindly supplied by Rhone-Poulenc (Vitry, France). Quinine hydrochloride was a generous gift from Sanofi-Winthrop (Gentilly, France). The structures are shown in Fig. 1. Stock solutions containing 500 μg/ml of each compound were prepared in methanol–water (50:50, v/v) and stored at −20°C. Working solutions of concentration 50 μg/ml for Q and 5 μg/ml for CQ, metabolites and I.S. were obtained by appropriate dilution of the stock standard solutions just before use; these solutions were used for drug-free plasma, erythrocytes and urine spiking. All standards were freshly prepared each day. Formate buffer (0.1 *M*, pH 9.2) was obtained by mixing equal volumes of 0.1 *M* ammonium formate solution and 0.1 *M* ammonia solution.

Apparatus

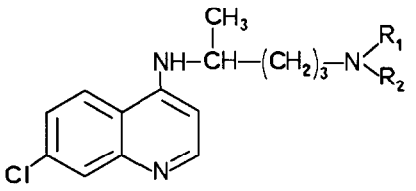
The HPLC equipment consisted of a Model 600 E multi-solvent delivery pump (Waters, Milford, MA, USA) connected to a refrigerated WISP 712 autoinjector (Waters). The fluorescence detector used was an RF 535 (Shimadzu, Kyoto, Japan) equipped with a 12-μl HPLC flow cell; the excitation wavelength was set at 325 nm (slit width 13 nm) and the emission wavelength at 375 nm (slit width 15 nm). The chromatographic response was recorded by Maxima 820 workstation software (Waters) running on a Power Mate SX Plus personal computer (NEC, Boxborough, MA, USA). The Maxima 820 chromatography workstation also included a system interface module (Waters) and a Pinwriter P6200 printer (NEC).

Chromatographic separations were performed at ambient temperature on an inert silica Inertsil column, 5 μm particle size (250 mm × 4 mm I.D.) (Interchim, Montluçon, France). An Inertsil guard column (10 mm × 4 mm I.D.) was placed between the injector and the analytical column. The solid-phase extraction pretreatment of the sample was carried out on a Bond Elut C_8 cartridge connected to a Vac Elut SPS 25 manifold (Prolabo, Paris, France).

Chromatographic conditions

The mobile phase was a mixture of two liquids, (A) acetonitrile and (B) methanol–25% ammonia solution (92.5:7.5, v/v), distributed by the gradient pump. The initial ratio of the mobile phase components (A/B) was 78:22 (v/v) and the flow-rate was set at 0.85 ml/min. The gradient elution conditions are given in Table I. Before analysis the liquids were filtered and degassed through a 0.5-μm FH filter (Millipore, Bedford, MA, USA) with a Pyrex filter holder. During assay, ammo-

Chloroquine



	R1	R2
Chloroquine	CH_2-CH_3	CH_2-CH_3
Monodesethylchloroquine	CH_2-CH_3	H
Bidesethylchloroquine	H	H
Hydroxychloroquine	CH_2-CH_2OH	CH_2-CH_3

Quinine

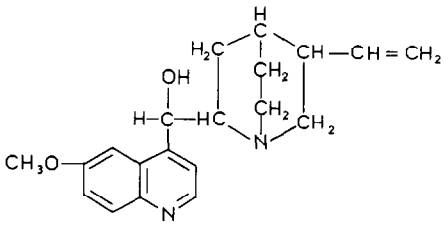


Fig. 1. Structures of chloroquine, its metabolites, quinine and internal standard.

nia evaporation was limited by keeping solution B at 4°C.

TABLE I

GRADIENT ELUTION CONDITIONS

Time (min)	Flow-rate (ml/min)	A (%)	B (%)	Curve[a]
0	0.85	78	22	–[b]
3	0.85	78	22	6
5	0.95	65	35	3
25	0.95	65	35	6
30	0.85	78	22	3

[a] Corresponding to gradient kinetics.
[b] No gradient kinetics.

Sample collection

Blood samples were provided from subjects undergoing a chemoprophylactic regimen of CQ (700 mg per week as base). Samples were also obtained from patients with acute attacks of malaria treated by intravenous injections of quinine (30 mg/kg per day). Whole blood was collected by venipuncture into heparinized tubes and centrifuged immediately at 1500 *g* for 15 min [5]. The upper two thirds of the plasma and the lower two thirds of the erythrocytes layer were separated, frozen as aliquots and stored at −20°C. Drug-free plasma erythrocytes and urine were frozen at −20°C until analysed. Calibration, recovery and precision measurements were subsequently made using these frozen specimens.

Extraction procedure

Extraction of drugs from plasma, erythrocytes haemolysed in distilled water (1:3, v/v) or urine diluted in distilled water (1:99, v/v) was carried out using a 3-ml Bond Elut C_8 cartridge pretreated successively with methanol (2 ml) and formate buffer (2 ml). To 1 ml of sample 100 μl of I.S. (5 μg/ml) were added and the mixture was passed on to the cartridge. The washing step was performed with 4 ml of buffer and 2 ml of methanol–buffer (50:50, v/v) successively.

Elution was carried out with 3 ml of methanol–ammonia solution (99:1, v/v). The eluate was evaporated to dryness under a stream of nitrogen in a borosilicate tube at 30°C. The residue was dissolved in 250 μl of the initial mobile phase [A–B (78:22, v/v)] by vortex-mixing and 50 μl were injected into the column.

Calibration procedure

Calibration based on peak-area ratio (CQ/I.S., MDCQ/I.S., BDCQ/I.S., Q/I.S.) was performed using spiked drug-free plasma, haemolysed erythrocytes and diluted urine, which were carried through the whole analytical procedure; calibration points were obtained by spiking biological samples with working standard solutions to achieve concentrations of 25, 100, 400 and 1000 ng/ml of CQ, MDCQ and BDCQ and 100, 1000, 10 000 and 20 000 ng/ml of Q. Five samples were run at each concentration. The peak-area ratio of drugs or metabolites to the I.S. was plotted against the corresponding concentration. A linear regression was performed in order to obtain the linearity, slope, intercept and correlation coefficient of each calibration graph. Calibration graphs were prepared with each analytical run.

Analytical recovery and assay precision

Absolute recovery was determined by comparing the peak areas obtained from plasma erythrocytes and urine samples spiked at three levels of each compound (100, 1000, 10 000 ng/ml Q and 25, 100 and 400 ng/ml CQ and metabolites) with those obtained by direct injection of the standards. Inter- and intra-assay precision were obtained by replicate assays of samples from pools of spiked plasma, erythrocytes and urine at three concentrations. The intra-assay precision was assessed at three concentrations from 30 samples. The inter-assay variation was determined over a period of two weeks with ten analysis of the same spiked biological fluids. The relative standard deviation [coefficient of variation (C.V.)] of the estimated concentrations were determined and used for the assessment of precision.

Limits of decision, detection and quantification

The limits of the method for all compounds were calculated from the analysis of drug-free plasma samples (n = 30) with average [M (blank)] and standard deviation [S.D. (blank)]. These lower limits are functions of the magnitude of the blank value and Massart *et al.* [17] described three levels, for decision [Lc = M(blank) + 3 S.D.(blank)], detection [Ld = M(blank) + 6 S.D.(blank)] and quantification [Lq = M(blank) + 10 S.D.(blank)] as quality criteria of an analytical method.

RESULTS

Selectivity

Fig. 2 shows (a) an HPLC profile of an extracted blank plasma with internal standard and (b–d) typical chromatograms obtained according to the described method from (b) spiked human plasma, (c) erythrocytes and (d) urine. The mobile phase provides a good resolution of the five compounds; the elution sequence and retention times are Q (8.5 min), OHCQ (11.5 min), CQ (15.6 min), BDCQ (19 min) and MDCQ (21.9 min).

Fig. 3 shows the chromatogram obtained from plasma collected from a patient under a CQ chemoprophylaxis regimen (700 mg per week) and suffering with an acute attack of malaria treated by intravenous injection of Q. No endogenous substances were found to interfere with the analysis in plasma, erythrocytes or urine. The potential interference with other drugs was examined; the method was shown to be free from chromatographic interferences from the other antimalarial drugs including proguanil and its metabolites (cycloguanil, 4-chlorophenylbiguanide), amodia-

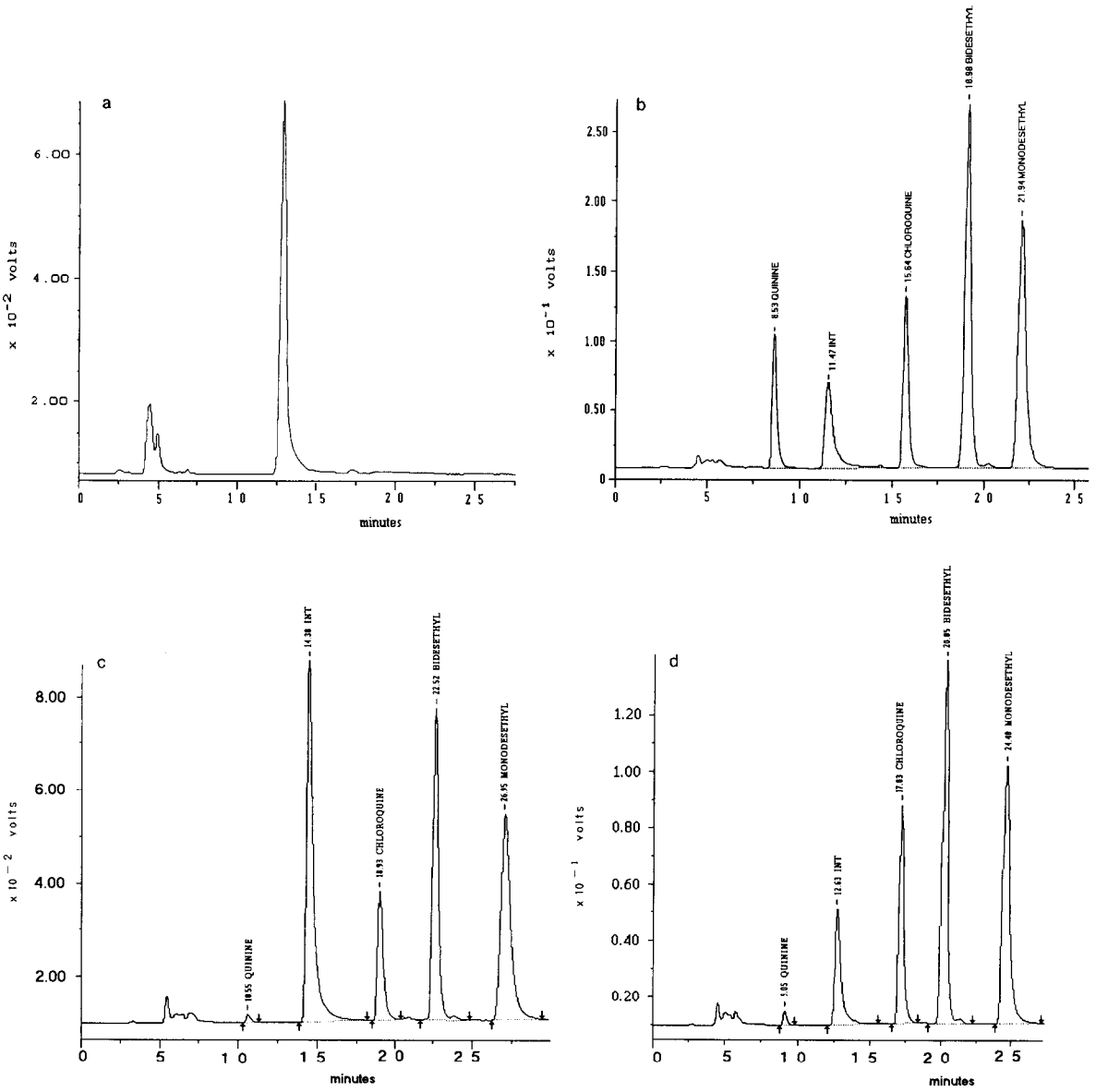


Fig. 2. HPLC profiles of (a) extracted blank plasma with internal standard, (b) spiked human plasma containing 400 ng/ml CQ, MDCQ, BDCQ and Q, (c) spiked human urine containing 100 ng/ml CQ, MDCQ, BDCQ and Q and (d) spiked human erythrocytes containing 400 ng/ml CQ, MDCQ, BDCQ and Q. INT = internal standard.

quine, halofantrine (retention time 4.5 min), mefloquine, pyrimethamine, sulphadoxine and other cinchona alkaloids, quinidine (the diastereoisomer of quinine) (retention time 7.7 min), cinchonine and cinchonidine. In order to evaluate possible interferences of Q metabolites with CQ, MDCQ and BDCQ assay, plasma samples from a patient treated only with Q were processed; none of them gave rise to interfering peaks.

Calibration graphs for drugs and metabolites

The ratio between the peak area of the drugs analysed and that of the I.S. was calculated and plotted against the concentrations of the drug

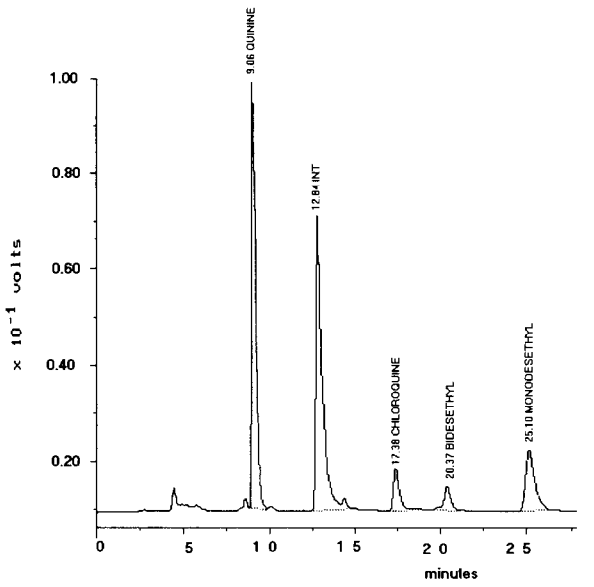


Fig. 3. Chromatogram of an extracted plasma from a patient under CQ chemoprophylaxis regimen and treated with Q. INT = internal standard.

tested after analysis of blank samples spiked with increasing concentrations of CQ, MDCQ, BDCQ (25, 100, 400 and 1000 ng/ml) and Q (100, 1000, 10 000 and 20 000 ng/ml) and a constant amount of I.S. Within these concentration ranges linear plots were obtained for the four compounds from the three biological fluids. The correlation coefficients (r^2) were greater than 0.999. The calibration functions were as follows: CQ, $y = 0.00292x - 0.00075$; MDCQ, $y = 0.00674x - 0.00301$; BDCQ, $y = 0.00721x + 0.03458$; Q, $y = 0.00014x + 0.00422$ (y = drug/I.S. peak-area ratio; x = concentration). Excellent linearity was exhibited over the concentration ranges 25–20 000 ng/ml for Q and 12.5–2000 ng/ml for CQ, MDCQ and BDCQ.

Analytical precision and accuracy

Intra-assay precision was evaluated by the analysis ($n = 30$) of pools of biological samples spiked with 25, 100 and 400 ng/ml CQ and metabolites and with 100, 1000 and 5000 ng/ml Q; the results are presented in Table II. The C.V.s were less than 7.1% for CQ and its metabolites and less than 6.9% for Q. Inter-assay precision data for ten spiked biological specimens evaluated during a two-week period are given in Table II; the day-to-day C.V.s were less than 6.6% for all compounds. These values demonstrate that the precision of the method is good over the range of concentrations studied.

Accuracy was calculated as the percentage difference between the amount of drug added to drug-free plasma and the amount of drug measured. For CQ, MDCQ and BDCQ the values were 2.1, 3.5 and 3.5%, respectively (100 ng/ml; $n = 5$) and for Q the value was 3.1% (1000 ng/ml; $n = 5$).

Analytical recovery

The absolute recoveries of CQ, MDCQ, BDCQ, Q and OHCQ were calculated by comparison of the peak area obtained after extraction from plasma erythrocytes and urine samples containing a known amount of the substance with the peak area obtained after direct injection of 50 μl of pure solution [methanol–water (50:50, v/v)] containing the same amount of each compound. Five analysis were performed at each level. The mean recoveries of the drugs from biological samples are given in Table III. The recovery of the I.S. averaged 90% ($n = 20$) at a concentration of 500 ng/ml.

Limits of decision, detection and quantification

The limits of the method were calculated from the analysis of thirty drug-free plasma samples. The results are given in Table IV. These limits are also valid for urine and erythrocytes. They are similar to those reported previously.

DISCUSSION

The HPLC method described here for the simultaneous determination of CQ and Q in human plasma, erythrocytes and urine is accurate, sensitive and selective. The precision is good with C.V.s always lower than 7.1% and the linearity is suitable over the whole range of therapeutic concentrations. The whole procedure requires about 30 min and the same time is necessary for pre-

TABLE II

INTRA- AND INTER-ASSAY PRECISION

Concentration (ng/ml)	Sample	C.V. (%)			
		CQ	MDCQ	BDCQ	Q
Intra-assay (n = 30)					
25	Plasma	4.8	5.0	5.2	
	Erythrocytes	4.7	4.5	4.5	
	Urine	2.7	3.3	3.4	
100	Plasma	4.1	5.0	5.4	5.9
	Erythrocytes	5.2	7.1	4.9	5.4
	Urine	2.7	2.4	2.4	5.9
400	Plasma	3.6	5.0	5.8	
	Erythrocytes	4.3	3.8	3.4	
	Urine	3.2	3.5	3.0	
1000	Plasma				6.9
	Erythrocytes				6.2
	Urine				3.7
5000	Plasma				4.4
	Erythrocytes				4.8
	Urine				3.7
Inter-assay (n = 10)					
25	Plasma	3.7	5.9	5.8	
	Erythrocytes	4.5	3.8	6.2	
	Urine	3.8	4.0	5.1	
100	Plasma	5.1	5.4	6.0	6.0
	Erythrocytes	5.2	5.9	6.2	5.9
	Urine	4.0	4.6	4.9	4.4
400	Plasma	4.2	3.9	3.3	
	Erythrocytes	6.3	5.4	5.9	
	Urine	4.8	4.3	4.1	
1000	Plasma				6.6
	Erythrocytes				4.3
	Urine				3.6
5000	Plasma				4.6
	Erythrocytes				4.8
	Urine				4.4

treatment of the biological sample. No chromatographic interferences from other commonly prescribed antimalarial drugs or endogenous compounds were observed. Advantages of the proposed method compared with other HPLC methods include the simultaneous determination of the two drugs Q and CQ, the use of solid-phase extraction on a Bond Elut C_8 cartridge and the choice of an appropriate internal standard; this internal standard (OHCQ) was selected because of its structural similarity to CQ, its suitable retention time and because it is not used as an antimalarial drug.

In conclusion, the HPLC method described here for the measurement of Q, CQ and metabolites either separately or simultaneously in biological fluids is suitable for routine analyses in clinical studies and drug monitoring.

TABLE III

RECOVERY OF THE ANALYTICAL METHOD

Concentration (ng/ml)	Sample	Mean recovery (%) (n = 5)			
		CQ	MDCQ	BDCQ	Q
25	Plasma	86.5	74.0	82.5	
	Erythrocytes	71.2	67.9	63.1	
	Urine	87.1	67.7	62.3	
100	Plasma	85.3	73.9	80.5	86.1
	Erythrocytes	77.4	69.6	64.6	81.2
	Urine	80.8	71.9	69.5	70.0
400	Plasma	88.5	78.1	82.5	
	Erythrocytes	83.5	77.4	69.6	
	Urine	78.4	75.4	70.8	
1000	Plasma				88.3
	Erythrocytes				80.7
	Urine				78.3
10 000	Plasma				88.6
	Erythrocytes				90.6
	Urine				79.5

TABLE IV

LIMITS OF DECISION, DETECTION AND QUANTIFICATION

Drug	Lc[a] (ng/ml)	Ld[a] (ng/ml)	Lq[a] (ng/ml)
Q	14.6	23.5	35.5
CQ	4.1	4.7	5.6
MDCQ	1.4	2.2	3.3
BDCQ	1.8	2.5	3.5

[a] For definitions, see text.

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REFERENCES

1 Y. Bergqvist and M. Frisk-Holmberg, *J. Chromatogr.*, 221 (1980) 119.
2 M. A. Staiger, P. Nguyen-Dinh and F. C. Churchill, *J. Chromatogr.*, 225 (1981) 139.
3 G. Alvan, L. Ekman and B. Lindström, *J. Chromatogr.*, 229 (1982) 241.
4 L. C. Patchen, D. L. Mount, I. K. Schwartz and F. C. Churchill, *J. Chromatogr.*, 278 (1983) 81.
5 E. Pussard, F. Verdier and M. C. Blayo, *J. Chromatogr.*, 374 (1986) 111.
6 S. Looareesuwan, N. J. White, C. Chanthavanich, G. Edwards, D. D. Nicholl, C. Bunch and D. A. Warrel, *Br. J. Clin. Pharmacol.*, 22 (1986) 31.
7 O. Walker, M. A. Salako, G. Alvan, O. Ericsson and F. Sjoqvist, *Br. J. Clin. Pharmacol.*, 23 (1987) 295.
8 K. Rauch and J. Ray, *J. Chromatogr.*, 430 (1988) 170.
9 P. Augustijns and N. Verbeke, *J. Liq. Chromatogr.*, 13 (1990) 1203.
10 J. F. Chaulet, C. Mounier, O. Soares and J. L. Brazier, *Anal. Lett.*, 24 (1991) 665.
11 C. Salvadori, R. Farinotti, A. Dauphin and G. Mahuzier, *Ann. Biol. Clin.*, 40 (1982) 667.
12 M. D. Edstein, J. Stace and F. Shann, *J. Chromatogr.*, 278 (1983) 445.
13 J. H. Galloway, I. D. Marsh and A. R. W. Forrest, *J. Anal. Toxicol.*, 14 (1990) 345.
14 A. R. Zoest, S. Wanwimolruk and C. T. Hung, *J. Liq. Chromatogr.*, 13 (1990) 3481.
15 V. Hellgren, T. Villen and O. Ericsson, *J. Chromatogr.*, 528 (1990), 221.
16 M. D. Edstein, A. Prasitthipayong, A. Sabchareon, T. Chongsuphajaisiddhi and H. K. Webster, *Ther. Drug Monitor.*, 12 (1991) 493.
17 D. L. Massart, B. G. M. Vandeginste, S. N. Deming, Y. Michotte and L. Kaufman, *Chemometrics: a Text Book*, Elsevier, Amsterdam, 1988, Ch. 7, p. 110.