ELSEVIER

Journal of Chromatography B, 666 (1995) 347–353

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Simple and specific reversed-phase liquid chromatographic method with diode-array detection for simultaneous determination of serum hydroxychloroquine, chloroquine and some corticosteroids

Pirkko Volin[a,b]

[a]*United Laboratories, P.O. Box 222, SF-00381, Helsinki, Finland*

[b]*Department of Chemistry, Organic Chemistry Division, University of Helsinki, P.O. Box 6, Vuorikatu 20, FIN-00014 Helsinki, Finland*

First received 17 August 1994; revised manuscript received 12 December 1994; accepted 19 December 1994

Abstract

This paper describes a simple, specific, and sensitive high-performance liquid chromatographic (HPLC) method using ion-pair reversed-phase chromatography with photodiode-array detection for the simultaneous determination of hydroxychloroquine (HCQ) and chloroquine (CQ) in serum samples from rheumatoid arthritis patients receiving either HCQ sulphate or CQ diphosphate. The assay is also applicable to the simultaneous determination of corticosteroids. The method consisted of two diethyl ether extractions of 1.0 ml of serum, to which two internal standards (2,3-diaminonaphthalene and 18-hydroxy-11-deoxycorticosterone) and 1.0 ml of 0.25 *M* sodium hydroxide had been added. After the organic phase was evaporated to dryness at 30–40°C under a stream of nitrogen, the extract was reconstituted with a 1:1 mixture of 0.1 *M* perchloric acid and methanol, an aliquot of which was injected on to the system. Peak-height ratios at different wavelengths ($A_{245/343}$, $A_{245/256}$, $A_{245/265}$ and $A_{245/275}$) were utilized as a method of assessing peak homogeneity. Some anti-inflammatory drugs which may be used for rheumatic disorders were shown not to interfere with the assay. The method provides selectivity by using diode-array detection at several wavelengths. The use of two internal standards not only compensates for losses during the sample manipulation but also prevents erroneous results in case of interference.

1. Introduction

Hydroxychloroquine sulphate and chloroquine phosphate have been used for the treatment of rheumatoid arthritis for a long time [1]. Recently, some patients with severe rheumatoid arthritis have been shown to benefit from high dose, intermittent methylprednisolone (MP) treatment [2–4]. Regular monitoring of serum concentrations of HCQ and CQ has proven to be important in preventing the occurrence of side effects [5,6]. Laaksonen et al. [5] showed safe serum concentrations of 0.8–0.9 μmol/l during

* Address for correspondence: Department of Chemistry, Organic Chemistry Division, University of Helsinki, P.O. Box 6, Vuorikatu 20, FIN-00014 Helsinki, Finland.

0378-4347/95/$09.50
SSDI 0378-4347(94)00584-2

CQ therapy and 1.4–1.5 μmol/l during HCQ therapy. If these limits were exceeded an increasing frequency of side effects, or even serious fatalities, could be expected [5,7]. Serum concentrations of administered corticosteroids have not yet been routinely monitored, but it might help to adjust the high dose individually [2].

A number of different HPLC methods have been proposed for the separate determination of HCQ, CQ and their metabolites in biological fluids [8–13]. In recent reports HCQ and CQ are separated as their enantiomers [14–16]. HPLC has been widely used also for steroid analysis, with normal-phase and reversed-phase techniques for the measurement of corticosteroids [17–23], but there have been no reports on the simultaneous determination of these drugs [24]. Many of the published analytical methods use detection at one wavelength only, but because of the similarity of the UV spectra of both HCQ, CQ, 2,3-diaminonaphthalene (the internal standard) and the steroid group, more information for the identification of the compounds was provided in this study by using absorbance ratios.

This work was performed to develop a simple HPLC method based on reversed-phase, gradient elution and diode-array detection. The method facilitates the simultaneous characterization of HCQ, CQ and co-administered synthetic corticosteroids from 1.0 ml of serum. In addition, the effects on adrenal function as indicated by serum cortisol can also be monitored.

2. Experimental

2.1. Apparatus

The determination was carried out using a Model 1090 liquid chromatographic system with a diode-array detector from Hewlett-Packard. The analytical column was Nova-Pak C_{18} (150 × 3.9 mm I.D.) of 4-μm particle size (Waters Chromatography Division of Millipore Corp., Milford, MA, USA). The column was protected with a 2-μm replaceable frit (Upchurch Scientific, Oak Harbor, WA, USA).

2.2. Reagents

Diethyl ether, acetonitrile and perchloric acid were obtained from Merck (Darmstadt, Germany), methanol was purchased from Orion (Helsinki, Finland), monobasic sodium phosphate was from Mallinckrodt (St. Louis, MO, USA) and heptanesulfonic acid monohydrate sodium salt was from Fluka Chemie (Buchs, Switzerland).

2.3. Drug standards and quality control

Hydroxychloroquine sulphate and chloroquine phosphate, which were U.S.P. reference standards (U.S.P. C., Rockville, MD, USA), were dried for 2 h at 105°C before use. Corticosteroids and 2,3-diaminonaphthalene were from Sigma (St. Louis, MO, USA).

Stock solutions of HCQ (67 mg/l), CQ (64 mg/l), and 2,3-diaminonaphthalene (1.5 g/l) were prepared in water and stored at 4°C for as long as six months. Stock solutions of corticosteroids in absolute ethanol (1.0 mg/ml) were stored in 4°C up to one year.

Working solutions of HCQ and CQ were prepared in water by diluting 1:100 from the stock solutions. Calibration standards were prepared in Lyphochek drug-free serum (Bio-Rad Labs., Richmond, CA, USA). For HCQ, the calibration standards had serum concentrations of 0.1, 0.2, 1.0, 2.0, and 5.0 μmol/l, and for CQ, 0.05, 0.1, 0.5, 1.0, 2.0 μmol/l. Calibration standards for steroids (MP acetate, MP, cortisol, prednisolone and prednisone), containing 50–2000 nmol/l, were diluted from stock solutions with water. The calibration standards were stable at −20°C as 5-ml aliquots for as long as three months. Working solution of the internal standards containing both 2,3-diaminonaphthalene (5.0 μg/ml) and 18-hydroxy-11-deoxycorticosterone (3.5 μg/ml) was prepared in water and kept at 4°C.

For intra- and inter-assay precision studies, concentrations of 1.5, 0.9 μmol/l and 400 nmol/l

for HCQ, CQ and the steroids, respectively, were stored at −20°C.

2.4. Procedure

The analytes were extracted twice on a rotary shaker for 15 min with 7 ml of diethyl ether from 1 ml of serum sample, added with 2,3-diaminonaphthalene (100 μl, 500 ng) and 18-hydroxy-11-deoxycorticosterone (350 ng) as the internal standards and 1 ml of 0.25 *M* sodium hydroxide. After the combined organic phases were evaporated under a stream of nitrogen at 30–40°C, 70 μl of a 1:1 mixture of 0.1 *M* perchloric acid and methanol was added to reconstitute the dried eluate, and 20 μl of the sample supernatant was injected onto the HPLC system.

2.5. Chromatographic conditions

The chromatographic conditions were as follows: The mobile phase consisted of 58 m*M* monobasic sodium phosphate buffer (7 g/l), to which 6 m*M* heptanesulfonic acid sodium salt was added (1.3 g/l), adjusted to pH 3.1 with concentrated phosphoric acid and acetonitrile–methanol (85:15), programmed to be delivered at a flow-rate of 1.0 ml/min at the following gradient: 100:0 (initial); 78:22 at 5 min; 70:30 at 17 min; 70:30 at 21 min; 65:35 at 30 min. For the simultaneous determination of prednisone and prednisolone, a slower gradient was used: 100.0 (initial); 83:17 at 5 min; 75:25 at 17 min; 70:30 at 21 min; 65:35 at 30 min. Before use, the mobile phase was filtered and degassed with helium. Analysis was carried out at ambient temperature.

3. Results and discussion

The present HPLC method enables the simultaneous determination of HCQ and CQ in serum of rheumatoid arthritis patients receiving either HCQ sulphate or CQ diphosphate (Fig. 1). The method can also be used to detect synthetic corticosteroids and endogenous cortisol

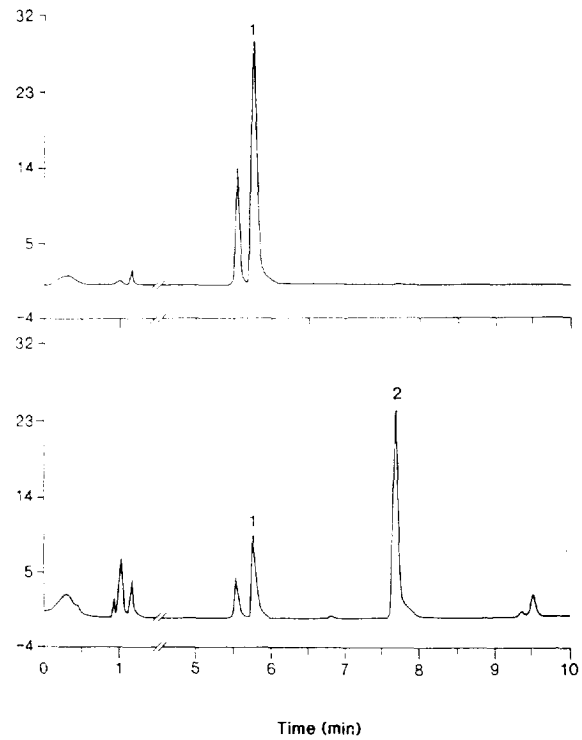

Fig. 1. Chromatogram of 1.0 ml serum from a patient receiving 200 mg of HCQ sulphate every day as a single dose. Peaks: 1 = HCQ (1.2 μmol/l) and 2 = 18-hydroxy-11-deoxycorticosterone (the internal standard). Mobile phase was isocratic methanol–acetonitrile–58 m*M* sodium dihydrogenphosphate containing 6 m*M* heptanesulphonic acid (4:22:74, v/v). Flow-rate was 1.8 ml/min and detection both at 343 nm (the upper chromatogram) and 256 nm.

concentrations. By using gradient elution the assay provides the determination of the compounds listed in Table 1 in a reasonable time. In the chromatograms which were obtained after extraction of 1.0 ml of blank serum, no additional peaks that could interfere with the determination of the compounds of interest were present. None of the commonly used drugs for rheumatoid arthritis tested (ibuprofen, indomethacin, phenylbutazone, and acetosalicylic acid) interfered with the present determination.

A chromatogram of a blank serum spiked with known concentrations of some compounds of interest is shown in Fig. 2. The drugs are eluted from an octadecylsilica column with methanol–acetonitrile–58 m*M* NaH_2PO_4 monohydrate

Table 1
Retention times and absorbance ratios of the compounds studied

Compound tested	Concentration (mg/l)	Retention time (min)	Absorbance-ratios			
			245/343	245/256	245/265	245/275
Triamcinolone	1.00	9.59		1.55	3.94	8.20
2,3-Diaminonaphthalene	0.26	10.71	7.71	1.90	3.74	3.45
HCQ	0.47	11.93	0.80	0.87	1.36	5.44
CQ	0.22	13.20	0.81	0.92	1.11	3.77
Prednisone	1.00	13.63		1.28	1.76	2.97
Prednisolone	1.00	13.76		1.18	1.52	2.30
Cortisol	1.00	14.37		1.29	2.53	6.38
Cortisone	1.00	14.73			3.14	8.45
18-Hydroxy-11-deoxy-corticosterone	0.87	15.85		1.18	1.95	3.45
MP	1.00	18.70		0.06	1.52	2.27
Fluprednisolone	1.00	19.17		1.28	1.94	3.62
Paramethasone	1.00	19.19		1.23	1.69	2.62
Betamethasone	1.00	19.45		1.38	1.81	2.21
Meprednisone	1.00	19.51		1.30	1.78	2.31
Dexamethasone	1.00	19.99		1.35	1.78	2.27
Corticosterone	1.00	20.80		1.28	2.05	2.76
Fluendrenolide	1.00	21.15		1.97	5.00	8.67
MP acetate	1.60	21.36		1.25	1.75	3.12
Fluocinolone acetonide	1.00	24.75		1.59	2.95	7.29
Progesterone	0.85	25.66		8.00	3.49	5.10
Fluoromethalone	1.00	26.15		1.40	1.81	2.40
17δ-Hydroxyprogesterone	0.98	29.26		1.39	3.13	11.5
Pregnenolone	0.85	>36				

containing 6 m*M* heptane sulfonic acid with the faster gradient. The baseline is smooth and the peaks are symmetrical as detected by UV absorption at 343, 256 and 245 nm. Under these conditions, prednisone and prednisolone were not very well separated at 13.7 min, but they could however be resolved by the slower gradient at 14.15 and 14.77 min, respectively.

The corticosteroids usually have maximum absorbance near 240 nm. For most steroids (MP acetate, MP, prednisolone, cortisol, cortisone, progesterone, 17-δ-hydroxyprogesterone and pregnenolone) 245 nm appears to be the optimal wavelength for monitoring under the conditions used. As has been shown in earlier studies, absorbance ratios can be easily reproduced even in complex samples at low concentrations [25]. Thus, interferences by metabolites or other compounds can be minimized or even eliminated. Because of the similarity of the UV spectra of the compounds in the two groups (group 1: chloroquine, hydroxychloroquine, 2,3-diaminonaphthalene and group 2: corticosteroids), peak purities were tested from chromatograms recorded at several wavelengths (343, 275, 265, 256 and 245 nm) using ratios of absorbances ($A_{245/343}$, $A_{245/256}$ for group 1, and $A_{245/265}$, $A_{245/275}$ for group 2) selected to provide the widest variation in the ratio values. In addition, co-analyzing HCQ and CQ with a pair of compounds that elute before and after the unknown compound and calculating the retention relative to both reference standards results in reasonably reproducible data. 2,3-Diaminonaphthalene turned out to be an excellent internal standard for all the compounds examined, being detectable at all the wavelengths used. 18-Hydroxy-11-deoxycorticosterone was added as an extra internal standard for both HCQ, CQ and corticosteroids because

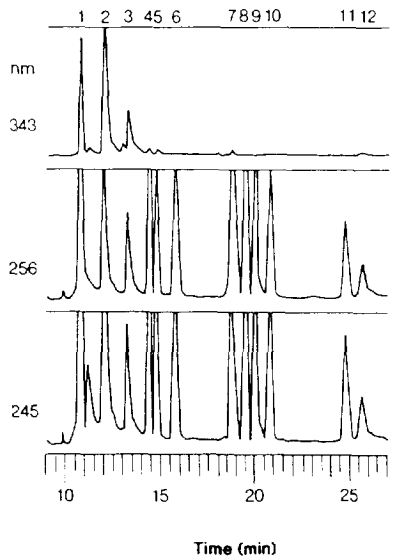

Fig. 2. Chromatogram of a spiked 1.0-ml serum extract containing 9 ng of HCQ, 4 ng of CQ, 15 ng each of the corticosteroid, and 20 ng each of the internal standards. Peaks: 1 = 2,3-diaminonaphthalene, 2 = HCQ, 3 = CQ, 4 = cortisol, 5 = cortisone, 6 = 18-hydroxy-11-deoxycorticosterone, 7 = MP, 8 = meprednisone, 9 = dexamethasone, 10 = corticosterone, 11 = fluocinolone acetonide, and 12 = progesterone.

of its proper retention time with baseline separation under the conditions used with no interference, neither from endogenous compounds nor the drugs studied. Absorbance ratios of the standards were averaged from triplicate runs (Table 1) and compared to those of the unknown peaks. This technique is a relatively simple way of determining peak purity of a specific drug that may have a retention time close to other drugs. For fully separated peaks the characteristic spectra can be determined easily and the absorbance ratio has been shown to be very reproducible [26]. However, in the case of severe peak overlap it is difficult to estimate the ratio value. For the true identification of an unknown solute, chromatograms were run under different conditions using methanol–tetrahydrofuran–58 m*M* NaH_2PO_4 monohydrate containing 6 m*M* heptane sulfonic acid (4:22:74) as an isocratic mobile phase. Because the elution order of the steroids in THF was usually different from that in acetonitrile, peak purity can be established, even when there would be a severe overlap of many compounds.

For HCQ and CQ the minimum detectable concentration was 2 ng/ml at 343 nm with a signal-to-noise ratio of 3. Detection sensitivities of 1–10 ng/ml at 245 nm were typical for the corticosteroids, which have high UV molar absorptivities. Calibration plots for the compounds of the standard mixture (HCQ, CQ, prednisone, prednisolone, cortisol, MP and MP acetate) were made at all the wavelengths used. Standard curves of corticosteroids, HCQ and CQ in drug-free serum exhibited excellent linearity over the concentration ranges of 25–2000 nmol/l, 0.1–10.0 μmol/l and 0.05–3.0 μmol/l, respectively. Correlation coefficients (r) for both internal standards ranged between 0.995 to 0.999, demonstrating that either of the two internal standards can be considered for quantitation. The method was found to be quite reproducible, as indicated by the low values of the coefficients of variation which were less than 6.0% for each steroid examined (MP acetate, MP, prednisone, prednisolone, and cortisol) over a concentration range of 50–1000 nmol/l and ranging from 5.2% (at 0.6 μmol/l) to 4.5% (at 1.5 μmol/l) for HCQ, and 5.6% (at 0.3 μmol/l) to 3.7% (at 0.9 μmol/l) for CQ within-run. The corresponding between-run values were less than 6.2% (at 400 nmol/l) for the steroids, 4.7% (at 1.5 μmol/l) for HCQ and 4.5% (at 0.9 μmol/l) for CQ. The recoveries of the extraction procedure for the drugs and the internal standards were determined by comparing the peak-heights of the corticosteroids, HCQ, CQ, and the internal standards obtained from extracted samples with those obtained by direct injection. Recoveries were higher than 85% at a concentration of 450 nmol/l, 0.6 μmol/l and 1.5 μmol/l for each of the compounds, respectively.

Water and acetonitrile possess little UV absorption between 200–400 nm and are therefore suitable components for the mobile phase. In the present study both basic and neutral compounds were separated. Acetonitrile–water as mobile phase was unsuccessful for the separation of both basic and neutral compounds. Acetonitrile–methanol (22:4) as an organic solvent added to

water gave good separations of the steroid mixtures. Under these conditions the basic compounds HCQ and CQ eluted with broad asymmetrical peaks with weak absorption. When phosphate buffer containing SDS as a counterion was used in the mobile phase instead of water, the retention times decreased resulting in narrower and non-tailing peaks. Because corticosteroids were non-ionized in the mobile phase used, their retention times remained about the same whether or not SDS was used. Solvent programming provided reasonable retention times for late eluting steroids (e.g. progesterone) while early eluting peaks remained well resolved. Another advantage of gradient elution was the very short re-equilibration time.

Acidic contaminants are often removed from steroid extracts by washing with a base, thereby leaving the steroids in the organic phase [18]. Mejer and Blanchard [27] studied the effects of serum prealkalinization, before extraction, on final cortisol values. Their results indicated that prealkalinization with 0.25 *M* NaOH eliminates noncortisol fluorescence from the serum extract without affecting the 11-hydroxycorticosteroids themselves. Decreasing the concentration of NaOH from 1 *M* to 0.25 *M* in this study resulted in a slight decrease in the HCQ and CQ values. On the other hand, increasing the concentration of NaOH to 1 *M* caused a decrease in corticosteroid recovery, especially for cortisone.

The method described was developed for rapid quantitation of serum HCQ and CQ concentrations from rheumatoid arthritis patients. If necessary, fractions containing these drugs or their metabolites can be collected and proportions of individual enantiomers further determined [14,15]. The potential of the present method to determine simultaneously corticosteroids from 1.0 ml of serum after simple extraction makes this method more versatile compared to those described previously. HPLC with diode-array detection can produce the quality of data needed to enhance confidence in the identity of an unknown compound. It provides very high resolution and when solvent programming is used, a large polar range of compounds can be analyzed in a single run. Patients may vary in response depending on their individual capacity to metabolize and excrete drugs. Altered drug disposition during disease or as a result of various interactions might necessitate dose adjustment in selected patients. The proposed method is simple, specific and reproducible for the simultaneous quantitative determination of several drugs from human serum used in the treatment of rheumatoid arthritis patients. It is also sensitive enough to monitor suppressed circulating cortisol concentrations.

References

[1] A.H. MacKenzie and A.L. Scherbel, *Clin. Rheum. Dis.*, 6 (1980) 545.
[2] G.J. Lawson, *J. Chromatogr.*, 342 (1985) 251.
[3] C.J. Needs, M. Smith, J. Boutagy, S. Donovan, D. Cosh, M. McCredie and P.M. Brooks, *J. Rheumatol.*, 15 (1988) 224.
[4] P.J. Hayball, D.G. Cosh, M.J. Ahern, D.W. Schultz and P.J. Roberts-Thomson, *Eur. J. Clin. Pharmacol.*, 42 (1992) 85.
[5] A.L. Laaksonen, U. Koskiahde and K. Juva, *Scand. J. Rheumatol.*, 3 (1974) 103.
[6] M. Frisk-Holmberg, V. Bergkvist, B. Domeij-Nyberg, L. Hellstrom and F. Jansson, *Clin. Pharmacol. Ther.*, 25 (1979) 345.
[7] P. Kintz, S. Ritter-Lohner, J.M. Lamant, A. Tracqui, P. Mangin, A.A.J. Lugnier and A.J. Chaumont, *Human Toxicol.*, 7 (1988) 541.
[8] P. Houzé, A. de Reynies, F.J. Baud, M.F. Benatar and M. Pays, *J. Chromatogr.*, 574 (1992) 305.
[9] M. Estadieu, A. Durand and A. Viala, *J. Anal. Toxicol.*, 13 (1989) 89.
[10] E. Pussard, F. Verdier and M.C. Blayo, *J. Chromatogr.*, 374 (1986) 111.
[11] R. Brown, R.M. Stroshane and D.P. Benziger, *J. Chromatogr.*, 377 (1986) 454.
[12] S.E. Tett, D.J. Cutler and K.F. Brown, *J. Chromatogr.*, 344 (1985) 241.
[13] R.G. Morris, *J. Chromatogr.*, 338 (1985) 422.
[14] A.J. McLachlan, S.E. Tett and D.J. Cutler, *J. Chromatogr.*, 570 (1991) 119.
[15] J. Iredale and I.W. Wainer, *J. Chromatogr.*, 573 (1992) 253.
[16] D.R. Brocks, F.M. Pasutto and F. Jamali, *J. Chromatogr.*, 581 (1992) 83.
[17] R.D. Toothaker, G.M. Sundaresan, Y.P. Hunt, T.Y. Goehl, K.S. Rotenberg, V.K. Prasad, W.A. Craig and P.G. Weling, *J. Pharm. Sci.*, 71 (1982) 573.
[18] F.J. Frey, B.M. Frey and L.Z. Benet, *Clin. Chem.*, 25 (1979) 1944.

[19] V.K. Prasad, B. Ho and C. Haneke, *J. Chromatogr.*, 378 (1986) 305.
[20] M. Hariharan, S. Naga, T. VanNoord and E.K. Kindt, *J. Chromatogr.*, 613 (1993) 195.
[21] J.O. De Beer, *J. Chromatogr.*, 489 (1989) 139.
[22] N.K. Hopkins, C.M. Wagner, J. Brisson and T.E. Addison, *J. Chromatogr.*, 577 (1992) 87.
[23] G.J. Samaan, D. Porquet, J.-F. Demelier and D. Biou, *Clin. Biochem.*, 26 (1993) 153.
[24] R.D. Armstrong, J. English, T. Gibson, J. Chakraborty and V. Marks, *Ann. Rheum. Dis.*, 40 (1981) 571.
[25] A.M. Krstulovic, P.R. Brown and D.M. Rosie, *Anal. Chem.*, 49 (1977) 2237.
[26] A.C.J.H. Drouen, H.A.H. Billiet and L. De Galan, *Anal. Chem.*, 56 (1984) 971.
[27] L.E. Mejer and R.C. Blanchard, *Clin. Chem.*, 19 (1973) 710.