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THE DETERMINATION OF CLIOQUINOL IN BIOLOGICAL MATERIALS BY EXTRACTIVE ALKYLATION AND GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Clioquinol, or 5-chloro-7-iodo-8-hydroxyquinoline (Vioform®), and the internal standard, 5,7-dichloro-8-hydroxyquinoline are extracted from biological material in the form of their tetrahexylammonium salts into dichloromethane, where, in the presence of a methylating agent, both clioquinol and the standard are spontaneously transformed into their O-methyl derivatives. These derivatives can be purified by base-specific extraction and subsequently determined by gas chromatography; concentrations down to 10 ng per sample may be assayed. The method is compared with a previously reported procedure based on the O-acetyl derivatives.

INTRODUCTION

Earlier methods for measuring unchanged clioquinol in biological material[1,2] have involved successive steps of extraction, formation of the O-acetyl derivative and gas–liquid chromatography, 5,7-dichloro-8-hydroxyquinoline being used as internal standard. The methodological improvement reported in this paper is achieved by using the principle of extractive alkylation introduced into quantitative drug assay by Ervik and Gustavii[3].

PRINCIPLE AND CONDITIONS

Clioquinol and the internal standard 5,7-dichloro-8-hydroxyquinoline (I) can be extracted in the form of their tetrahexylammonium salts (II) from aqueous alkaline medium into a dichloromethane phase, where, in the presence of iodomethane (or another methylating agent, such as dimethyl sulphate), the O-methyl derivatives (III) of clioquinol and the standard are formed almost spontaneously as outlined in Fig. 1. The over-all yields of the procedure are dependent on the pH of the aqueous phase, as demonstrated in Fig. 2. The yields of the alkylation reaction alone are about 88% (as determined by using ^{14}C-labelled compounds). The structures of the O-methyl derivatives have been verified by mass spectrometry. For purification, the derivatives can be extracted from the organic solvent into acidic aqueous phase at pH $\simeq$ 0 and can be re-extracted with hexane at a pH above 2 as shown in Fig. 3. The methyl

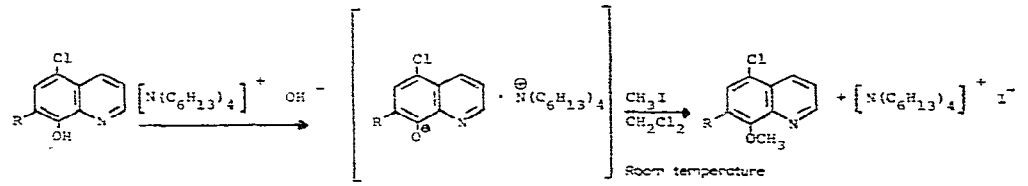

Fig. 1. Formation of the methyl derivatives of clioquinol (I; R = I) and internal standard (I; R = Cl).

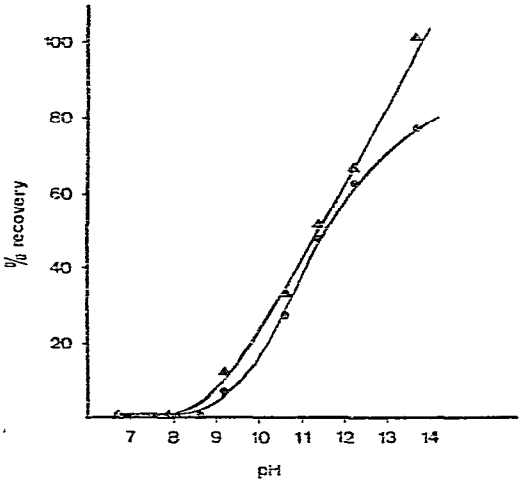

Fig. 2. Dependence on reaction pH of the recovery of clioquinol (●—●) and internal standard (▲—▲), as methyl derivatives, from plasma. 600 ng of clioquinol and 960 ng of internal standard in 0.5 ml of porcine plasma were each processed, and the total yield of methyl derivatives was determined by gas–liquid chromatography.

derivatives have excellent gas chromatographic properties and can be measured sensitively by use of an electron-capture detector.

It is known that, in animals and in man, clioquinol is transformed into hydrophilic conjugates, which, on hydrolysis, yield unchanged clioquinol[4,5]. To ascertain whether or not clioquinol conjugates in blood plasma are hydrolysed under the conditions of extractive alkylation, plasma samples from *in vivo* experiments were analysed for unchanged clioquinol both by the procedure described in this paper and by a modification that involved separate extraction of clioquinol and the standard at pH 7 with dichloromethane–diethyl ether (1:4). The results were found to be in good agreement, showing that unintended cleavage of clioquinol conjugates is not likely to be caused by the extractive-alkylation process.

The extractive-alkylation technique can be used not only for analysis of plasma and urine, but also for assaying unchanged clioquinol in tissue homogenates; some of the conditions under which plasma and tissue can be analysed are as follows.

For determinations in plasma, no more than 0.5 ml of sample should be used; larger amounts tend to change the slope of the calibration curve.

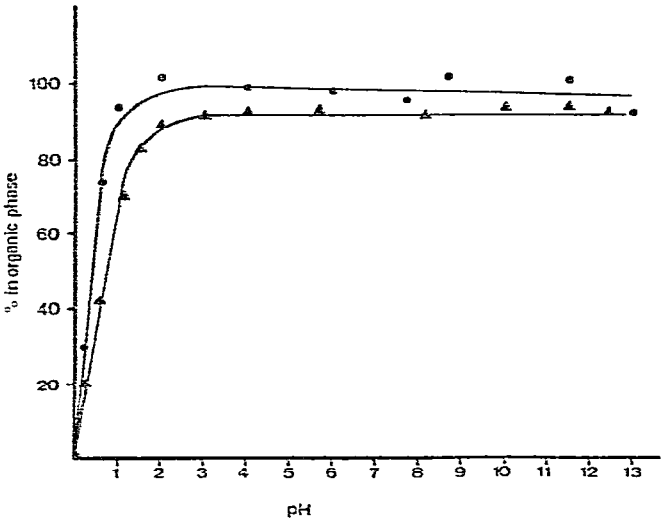

Fig. 3. Partition of O-methylated clioquinol (●—●) and O-methylated standard (▲—▲) between hexane and buffered aqueous phases as a function of the pH of the aqueous phase.

Tissue samples should be processed by preparing an aqueous homogenate (up to 500 mg per 5 ml), up to 1 ml of which may be subjected to the procedure described for plasma. The calibration curves prepared with plasma and with tissue homogenate are equivalent.

The determination of clioquinol in fat samples requires a modified procedure. Instead of preparing an aqueous homogenate, a dichloromethane homogenate is prepared using the same quantities as for tissues. An aliquot (1 ml) of the dichloromethane homogenate is added to the reaction mixture, which is prepared in the same way as for plasma. A calibration curve prepared with fat must be used in the analysis of fat samples, as the slopes of the calibration curves prepared with fat and with plasma are not the same.

EXPERIMENTAL

Reagents and solvents

All reagents and solvents were of analytical grade and were tested for purity by carrying out blank runs.

Extraction and derivatisation

From 0.1 to 0.5 ml of plasma is mixed with 0.5 ml of internal standard (500 ng of 5,7-dichloro-8-hydroxyquinoline) in 0.1 *N* hydrochloric acid, 2 ml of 2 *N* sodium hydroxide and 0.1 ml of 0.05 *M* tetrahexylammonium hydrogen sulphate (M.W. 451), and the mixture is shaken (at 80 rpm on a mechanical rotary shaker) with 2 ml of 1 *M* iodomethane in dichloromethane for 30 min at room temperature and then centrifuged. A maximum aliquot of the organic phase is evaporated to dryness in a stream of nitrogen, and the residue is dissolved in 2 ml of hexane and shaken with 1.5 ml of 5 *M* perchloric acid for 10 min at 150 rpm. To 1 ml of the acidic phase are added 4 ml of saturated trisodium phosphate solution in water, and the mixture is

shaken with 2 ml of hexane for 5 min at 150 rpm: a 5-μl aliquot of the final organic phase is injected into the gas chromatograph.

Gas chromatography

The instruments used were a Pye Unicam Model 74, Series 104, gas chromatograph with a pulsed (150 μsec) electron capture detector (^{63}Ni; 10 mCi), an Autojector S4 automatic injection unit and an Infotronics Model 208 integrator. The columns used were Pyrex glass, 5 ft. $\times$ 2 mm I.D., packed with 3% JXR on Gas-Chrom Q, the carrier gas was nitrogen (30 ml/min) and the temperatures were: injection port 200°; column oven 185° and detector 300°.

Three typical chromatograms obtained with extracts from human plasma are shown in Fig. 4.

Calibration curves

In order to calibrate the relative over-all yield of clioquinol in relation to the standard, plasma samples with known concentrations were analysed, and the mea-

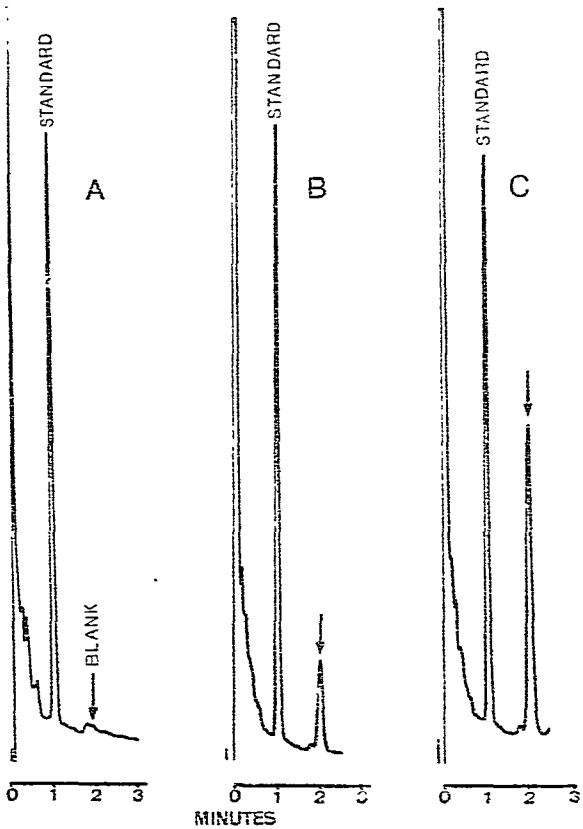

Fig. 4. Gas chromatograms obtained from 0.5-ml plasma samples containing no clioquinol (A), 50 ng of clioquinol (B) and 200 ng of clioquinol (C); each sample contained 250 ng of internal standard. The amounts injected were about 1‰ of those originally present in the sample. The arrows indicate the peak for the methyl derivative of clioquinol.

sured peak area ratio was plotted against the amount of clioquinol originally present in the sample. To demonstrate that clioquinol can be determined by this method even in samples of fat, the same calibration process was carried out with porcine fat. The respective calibration curves are shown in Fig. 5.

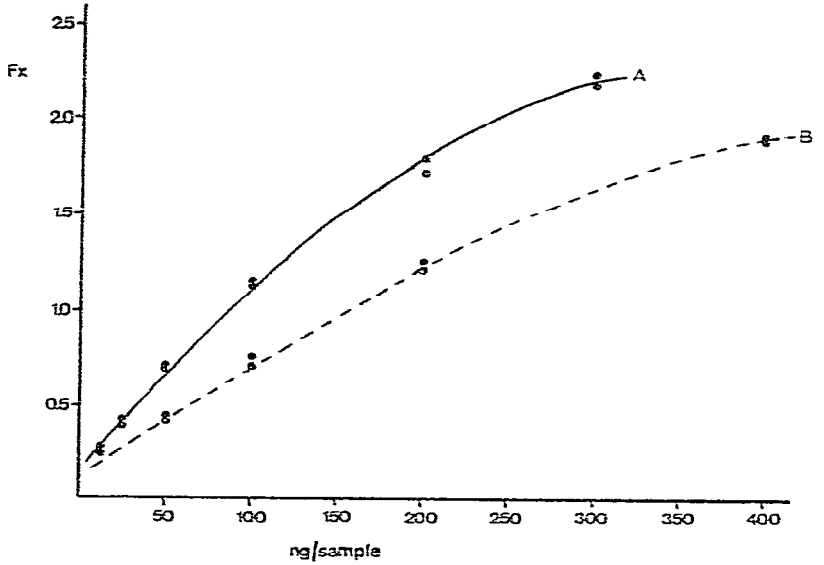

Fig. 5. Calibration curves for clioquinol in (A) 100 mg of porcine fat and (B) 0.2 ml of human plasma. Each sample contained 5,7-dichloro-8-hydroxyquinoline as internal standard (500 ng per sample of fat; 300 ng per sample of plasma). *Fx* is the ratio of the peak area for the clioquinol derivative to that for the internal standard derivative.

Sensitivity and precision

The method was tested by analysing prepared samples of plasma and tissue (canine liver) homogenates containing clioquinol concentrations unknown to the analyst. The results in Table I and II show that the sensitivity and precision are sufficiently good to permit the technique to be used for determining clioquinol at the levels reported for pharmacokinetic studies[1].

In addition, a number of samples were analysed by the proposed method in comparison with the O-acetylation method; the results are shown in Table III.

TABLE I

DETERMINATION OF CLIOQUINOL IN 0.5-ml SAMPLES OF HUMAN PLASMA

Clioquinol concentration (ng/ml)		*Number of determinations*	*Standard deviation (ng/ml)*	*Coefficient of variation (%)*
Present	*Found (mean)*			
25	23	3	1.0	4.3
50	46.4	8	4.3	9.4
100	102.1	8	2.9	2.8
200	203.9	9	4.7	2.3
400	390.3	6	22.6	5.8

TABLE II

DETERMINATION OF CLIOQUINOL IN 100-mg SAMPLES OF CANINE LIVER

Clioquinol concentration (ng/100 mg)		*Number of determinations*	*Standard deviation (ng/100 mg)*	*Coefficient of variation (%)*
Present	*Found (mean)*			
25	24	2	1.0	4.2
50	52.5	2	3.5	6.7
100	98.4	5	3.0	3.1
200	200.0	4	5.5	2.7
400	391.0	4	6.7	1.7
500	520.0	1	—	—

TABLE III

COMPARISON OF RESULTS FOR CLIOQUINOL IN 0.1-ml SAMPLES OF HUMAN PLASMA BY ACETYLATION AND BY EXTRACTIVE ALKYLATION

Samples contained 300 ng of internal standard for the acetylation method and 250 ng for the extractive alkylation method.

Clioquinol present (ng/0.1 ml)	*Clioquinol found (ng/0.1 ml)*	
	Acetylation method	*Extractive alkylation method*
10.4	12.5	11.8
	11.3	10.7
24.8	28.0	24.8
	30.0	24.1
53.6	62.0	51.8
	64.0	52.5
166.7	172.0	153.5
	192.0	162.0
375.0	392.0	370.0
	406.0	368.0

TABLE IV

COMPARISON OF RESULTS FOR UNCHANGED CLIOQUINOL IN 100-mg SAMPLES OF FAT BY TWO METHODS

Clioquinol present (ng)	*Clioquinol found (ng)*		*Deviation (%)*	
	Extractive alkylation	*Method of Tamura et al.*[2]	*Extractive alkylation*	*Method of Tamura et al.*[2]
125	125	150	0.0	+20.0
	130	125	+ 4.0	0.0
	115	135	− 8.0	+ 8.0
250	245	278	− 2.0	+11.2
	275	270	+10.0	+ 8.0
	270	278	+ 8.0	+11.2

The method of Tamura *et al.*[2] was used to estimate clioquinol in fat biopsy samples, and the results were compared with those of our technique; Table IV shows that agreement between the results of the two procedures was good.

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