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Note

Extractive alkylation of biological samples of clioquinol or chloroquinaldol and determination by electron capture gas chromatography

PER HARTVIG and CHRISTINA FAGERLUND

Department of Pharmacy, University Hospital, Fack, S-750 14 Uppsala (Sweden)

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The two 8-hydroxyquinolines, clioquinol (5-chloro-7-iodo-8-quinolinol) and and cloroquinaldol (5,7-dichloro-2-methyl-8-quinolinol), which are frequently used as antibacterial and antimycotic agents, are responsible for very severe side-effects after oral administration. Despite the widespread use of these compounds, few methods exist for their determination in biological samples[1–3], and none of these is capable of measuring concentrations below 50 ng/ml.

Recently a method appeared which uses extractive methylation of clioquinol before analysis by gas chromatography with electron capture detection (GC–ECD)[4]. Quantitation of the methyl ether derivative down to 10 ng/ml of biological sample was claimed. However, neither the poor stability of clioquinol in plasma or urine samples, nor the rapid decomposition of the methyl derivative in heptane solution was considered. Furthermore, the reaction conditions would cause pronounced hydrolysis of the methylating reagent owing to a high co-extraction of hydroxide ions into the organic phase.

In the present method tetrabutylammonium is used as counter-ion for the phenolate as ion-pair, which is extracted into methylene chloride where methylation occurs. The influence of pH on the reaction has been studied with respect to time and degradation of formed derivative. The highly selective electron capture response enables determinations directly from urine or plasma samples with a sensitivity equal to that of the previous method[4].

EXPERIMENTAL

Gas chromatography

A Pye GCV gas chromatograph equipped with a flame ionization detector (FID) and a ^{63}Ni electron capture detector (ECD) operated in the constant current mode was used in the evaluation of reaction conditions and in the analysis of biological samples, respectively. The glass column (150 × 0.2 cm) was filled with 3% OV-17 on Gas Chrom Z 80–100 mesh and operated at 215°. Injector and detector temperatures were maintained at 290 and 300°, respectively. The flow-rate of nitrogen carrier gas was 30 ml/min.

Reagents and chemicals

Tetrabutylammonium (0.1 *M*) was prepared by neutralization of tetrabutylammonium hydrogen sulphate (AB Hässle, Mölndal, Sweden) and purified by washing with equal volumes of methylene chloride four times and heptane twice. The tetrabutylammonium solution was then diluted to volume with phosphate buffer (pH 11, $\mu = 1$).

Methyl iodide, methylene chloride and heptane (Uvasol quality) were obtained from E. Merck (Darmstadt, G.F.R.). A saturated solution of silver sulphate in water was used.

Chloroquinaldol was used as internal standard in the determination of clioquinol and *vice versa*. They were dissolved and diluted to 1 μg/ml with buffer. Fresh solutions of clioquinol were prepared each day. β-Glucuronidase was obtained from Sigma (St. Louis, Mo., U.S.A.).

Methods

To evaluate the reaction conditions, clioquinol and chloroquinaldol (1 mg/ml) were dissolved in methylene chloride (1 ml) together with tetracosane (0.5 mg/ml) as internal standard. After addition of methyl iodide, the solution was shaken with 2 ml of 0.1 *M* tetrabutylammonium in phosphate buffer. The reaction was quenched by washing the organic phase with 1 ml of 1 *M* phosphoric acid, and 1 μl of the organic phase was injected into the gas chromatograph equipped with FID. The height ratio of formed derivative to internal standard was then calculated.

To determine the minimum detectable concentration, a solution of the derivatives of clioquinol and chloroquinaldol, prepared in the mg/ml range, was diluted and 10 pg injected into the gas chromatograph with ECD[5].

The plasma or urine sample (0.1 or 0.5 ml) was made alkaline with two drops of 5 *M* sodium hydroxide, and 0.1 ml of internal standard solution was added. To this solution were added 1 ml of tetrabutylammonium solution, 0.1 *M* in buffer, and sufficient water to make up to 2 ml. This was shaken for 30 min with 1 ml of methylene chloride and 100 μl of methyl iodide. After centrifugation for 15 min at 2500 rpm, as much as possible of the organic phase was transferred to another tube, 0.5 ml of heptane was added and methyl iodide and methylene chloride were completely evaporated in a stream of nitrogen. Another 1 ml of heptane was added and the organic phase shaken for 10 min with 1 ml of saturated silver sulphate solution. Some microlitres of the organic phase were injected into the gas chromatograph.

A standard curve was prepared by treating known amounts of the actual 8-hydroxyquinoline in plasma or urine according to the above procedure.

RESULTS AND DISCUSSION

Reaction conditions

Rapid consumption of alkylating reagent in extractive alkylation procedures at pH > 12 with the use of tetrahexyl- or tetrapentylammonium ion has been observed by Gyllenhaal[6]. The reduction in reagent concentration decreases the reaction rate and hence the yield of derivative is low. As the consumption is due to the co-extraction and alkylation of buffer anions, the use of tetrabutylammonium as counter-ion is strongly recommended.

For a short reaction time the acid should be completely ionized. The time course of the methylation of clioquinol and chloroquinaldol is given in Figs. 1 and 2, which show that a pH greater than 10 is required for rapid reaction. The highest reaction rate was observed at pH 12 although the yield decreased, most rapidly for clioquinol, over prolonged reaction times. To avoid this effect a pH value of 11 was used. The methylation was completed in 30 min, and the yield remained constant for more than 3 h. The choice of buffer system was also emphasized by Gyllenhaal[6].

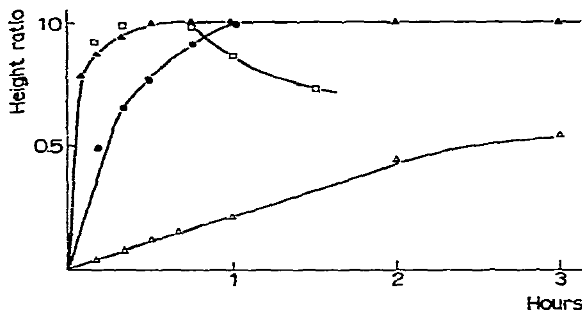

Fig. 1. Time course of the extractive methylation of clioquinol. Organic phase: methylene chloride with 10% of methyl iodide. Aqueous phase: tetrabutylammonium ion, 0.1 *M* in phosphate buffer (μ = 1). □, pH 12; ▲, pH 11, ●, pH 10; △, pH 8.

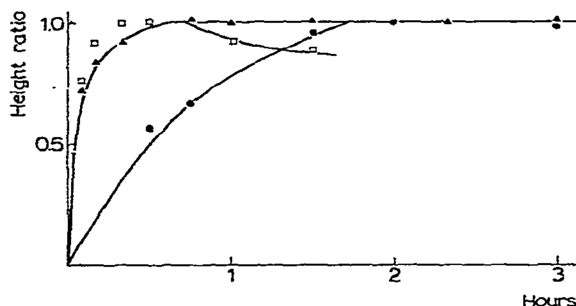

Fig. 2. Time course of the extractive methylation of chloroquinaldol. Organic phase: methylene chloride with 10% of methyl iodide. Aqueous phase: tetrabutylammonium ion, 0.1 *M* in phosphate buffer (μ = 1). □, pH 12; ▲, pH 11; ●, pH 10.

After conversion into the methyl derivatives the 8-hydroxyquinolines are amenable to GC–ECD. The presence of two halogen atoms renders the compounds highly responsive to electron capture. The minimum detectable concentrations of clioquinol and chloroquinaldol as methyl derivatives were 0.5 and 1.6×10^{-16} mole/sec, or corresponding to an injected amount of 0.5 pg of clioquinol on a column with 1500 theoretical plates and with a retention time of 4 min. A reaction time > 2 h was required with 2% of methylating reagent, whereas *ca.* 20 min was necessary with 20%. In practice, 10% of methyl iodide was used.

Injection of the reaction mixture directly into the gas chromatograph was not possible as methyl iodide and methylene chloride seriously disturbed the ECD. After addition of heptane these were easily removed by evaporation. Tetrabutylammonium

iodide, formed as a by-product, was removed using a saturated solution silver sulphate in water[7].

Determination in biological samples

The stability of the two 8-hydroxyquinolines was studied in urine and plasma samples at room temperature. The degradation was measured against an inert standard (Mirex®), and the results are given in Table I. The stability of clioquinol was extremely poor and only 50% remained after 3 days. Samples stored in a refrigerator showed no degradation after 7 days. For minimum degradation the samples were frozen immediately after sampling and were analysed not more than 3 days later. Clioquinol was also unstable in buffer solution at pH 7, even if stored in a dark room. A similar degradation of the methyl derivative of clioquinol in heptane solution was observed. Chloroquinaldol was found to be stable under the above conditions.

TABLE I

STABILITY OF CLIQUINOL AND CHLOROQUINALDOL

Determination by GC–ECD by comparison to inert standard (Mirex®). Concentration of 8-hydroxyquinoline: $5 \cdot 10^{-6}$ M (1 μg/ml). Values are the height ratio to the internal standard.

Day	*Plasma*		*Urine*		*Phosphate buffer (pH7)*		*Derivative in heptane*	
	Clioquinol	*Chloroquinaldol*	*Clioquinol*	*Chloroquinaldol*	*Clioquinol*	*Chloroquinaldol*	*Clioquinol*	*Chloroquinaldol*
0	1.00	1.02	1.00	0.99	1.00	1.00	0.99	1.01
1	0.88	1.05	0.68	1.00	0.68	1.05	0.82	1.02
2	0.66	1.08	0.62	—	0.56	1.00	0.53	0.95
3	0.48	1.00	0.50	0.96	0.36	0.96	0.40	0.96
6	0.36	0.98	0.49	1.02	0.24	0.93	0.10	0.98

The method has been used to determine clioquinol and chloroquinaldol in plasma and in urine. The main fraction of the 8-hydroxyquinolines is excreted in the urine as conjugated metabolites. The selectivity of the extractive alkylation procedure to the conjugated metabolites was previously verified[4]. The total amount of the compounds in urine was determined following enzymatic hydrolysis with β-glucuronidase. Interference from other metabolites is not likely.

Rectilinear standard curves through the origin were obtained for the two compounds in the range 25–150 ng added to plasma or urine samples. The method was quantitated down to 10 ng of clioquinol in a 0.5 ml plasma sample. The relative standard deviation was 6.6% for 25 ng of clioquinol in 0.5 ml of plasma ($n = 8$).

The clinical applications of this study are reported elsewhere[8].

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