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ELECTROPHORETIC STUDIES OF CLIOQUINOL BINDING TO HUMAN SERUM PROTEINS

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Abstract—The binding of radioisotope-labeled clioquinol to human serum proteins *in vitro* was studied by means of agarose gel electrophoresis. The technique gave a satisfactory separation of free and bound forms of clioquinol without a significant interaction between clioquinol and the supporting medium even at considerably high concentrations of the drug. The clioquinol binding proteins in serum were identified as albumin and lipo-proteins. Clioquinol was bound to albumin at an equimolar ratio with an apparent binding constant of $5.6 \times 10^4\ M^{-1}$. When the amount of clioquinol exceeded the binding capacity of albumin, lipoprotein classes of very low density, low density and high density were capable of serving as auxiliary carrier proteins of clioquinol.

The metabolic studies on clioquinol (5-chloro-7-iodo-8-quinolinol) were limited in number before its significant absorption from the gastrointestinal tract was demonstrated by several workers [1–3]. The studies of clioquinol binding to human serum proteins were initiated in our laboratory in view of the importance of protein binding for the absorption, tissue distribution and excretion of the drug and also in view of the consideration that the modification of clioquinol binding due to changes in the nature or amount of its binding proteins and in the serum level of other interfering substances in disease states would alter the above physiological processes and hence the action of the drug after absorption.

The methods employed for the studies of clioquinol binding, such as gel filtration [4], equilibrium dialysis [4–6] or paper electrophoresis [7], were found in our preliminary experiments to show a considerable interference between clioquinol and the supporting medium particularly at higher clioquinol concentrations, as was suggested also by others [4]. The difficulties were overcome by using agarose gel as the supporting medium in electrophoretic separation of protein-bound clioquinol. The present report describes the binding of clioquinol to human serum proteins under normal and pathological conditions by means of agarose gel electrophoresis with a sufficiently high concentration of clioquinol. The results indicate that, besides albumin, lipoproteins can serve as clioquinol carrier proteins in serum. Part of this work has been reported in abstract form [8].

MATERIALS AND METHODS

Either [7-^{131}I]clioquinol (0.41 mCi/mg) prepared by Daiichi Radioisotope Laboratories, Ltd., Tokyo, or [2,4-^{14}C]clioquinol (13.6 μCi/mg) donated by Dr. Goro Urakubo, National Institute of Hygienic Sciences, Tokyo, was used after dissolving them in 0.1 N NaOH to give a concentration of 0.15 mg/ml, placing them in dark bottles and freezing at −20°. Under these conditions, the labeled clioquinol gave a single spot of radioactivity with a positive organic iodine reaction on paper chromatography [9, 10]. Ten μl of 0.15 mg/ml of clioquinol and 100 μl of fresh serum after appropriate dilution with a barbital buffer, ionic strength 0.025 and pH 8.6, were mixed and kept for 10 min at room temperature. Control studies confirmed that the amount of NaOH present in the clioquinol solution did not alter the pH of variously diluted serum. Eight-μl aliquots of the mixture for [^{131}I]clioquinol or 20 μl for [^{14}C]clioquinol were applied on a 1% agarose gel plate prepared in the barbital buffer (ionic strength 0.025 and pH 8.6). Electrophoresis was carried out for 90 min at room temperature with a constant voltage of 110 V/10 cm using a barbital buffer, ionic strength 0.05 and pH 8.6. The agar plate was fixed by drying in a warm air stream at 45° immediately after electrophoresis. The radioactivity was located either by scanning with a thin-layer chromatogram scanner, model JTC-201, or by an autoradiographic technique, and the proteins and lipoproteins were stained with Ponceau 3R and acetylated Sudan Black B respectively. Lipoprotein fractions were separated by the ultracentrifugal method of Yasugi and Honma [11], desalted with Collodion bags (Sartorius-membrane filter GmbH) and studied for clioquinol binding by the electrophoretic method with a 0.4% agarose–0.12% agar in place of 1% agarose.

Sera were separated from blood samples collected from patients with various diseases after an overnight fast. The sera similarly obtained from healthy subjects served as controls. The concentrations of serum bilirubin, total protein and its fractions, nonesterified fatty acids (NEFA) and triglycerides were determined by routine laboratory methods. Crystallized human albumin was obtained from Miles Laboratories, Inc. All experiments with radioisotope-labeled compounds were carried out at the Radioisotope Research Center, Okayama University Medical School.

RESULTS

In agarose gel electrophoresis of [^{131}I]clioquinol mixed with sera from healthy subjects, a single peak

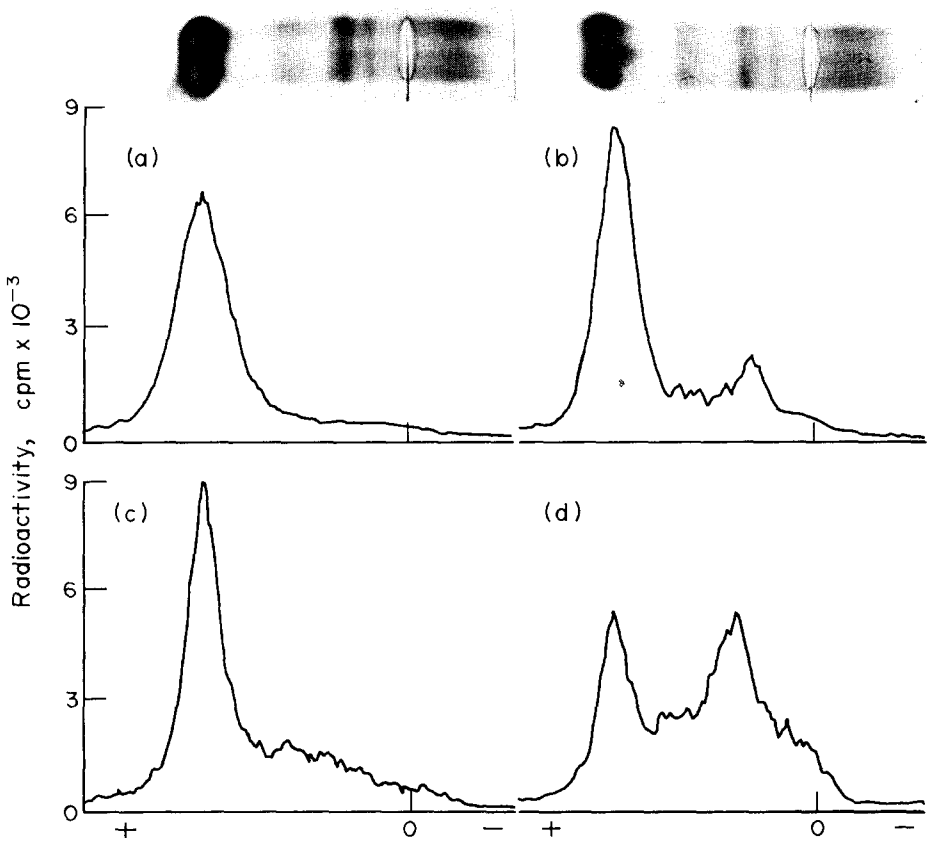

Fig. 1. Electrophoretic patterns of radioactivity and protein staining with sera from a healthy subject and from a case with hypoalbuminemia. Panels a and c: serum from a healthy subject (Case 18 in Table 1); panels b and d: serum from a case of metastatic liver cancer with hypoalbuminemia (Case 1 in Table 1). The concentrations of [^{131}I]clioquinol in serum were: in panels a and b, 15 μg/ml; and in panels c and d, 60 μg/ml. The concentrations of total protein and albumin in these sera are given in Table 1. The photographs at the top show the patterns of protein staining for a and b; 0 denotes the origin.

of radioactivity at the position of albumin migration was found when the clioquinol concentration was 15 μg/ml of serum (Fig. 1a). By increasing the clioquinol-serum ratio to 60 μg/ml, using diluted serum and keeping the clioquinol concentration constant, an additional shoulder or peak of radioactivity was produced in the α_2-β globulin region (Fig. 1c). Omitting serum from the mixture gave a radioactivity peak remaining at the origin (cf. Fig. 2d). Among patients having either decreased albumin or increased bilirubin, NEFA or triglyceride concentration, and combinations thereof, Cases 1, 2 and 3 in Table 1 gave an extra-albumin peak of radioactivity even at a clioquinol concentration of 15 μg/ml of serum (Fig. 1b), and the peak height was more pronounced when the clioquinol concentration was increased to 60 μg/ml of serum (Fig. 1d). These results suggest that clioquinol binds primarily to albumin and then to other proteins in the α_2-β region as the amount of clioquinol exceeds the binding capacity of albumin. It is also apparent from the results given in Table 1 that the decreased concentration of albumin rather than the increased levels of serum bilirubin, NEFA and triglycerides, which might interfere with clioquinol binding to albumin, is primarily important for the increased height of the extra-albumin peak in these patients. The clioquinol displacement by NEFA may have a small but significant contribution to the increased extra-albumin binding, since a small α_2-β peak was produced with a control serum mixed with [^{131}I]clioquinol (60 μg/ml) and sodium palmitate at a final concentration of 2.21 meq/l. Unconjugated bilirubin tested at a concentration of 8.1 mg/dl had no such effect. The electrophoretic patterns of a clioquinol-serum mixture at a ratio of 60 μg/ml in studied cases are summarized in Table 1. In cases with increased serum lipid levels due to obstructive jaundice and nephrotic syndrome, the main extra-albumin peak was found in the β-globulin region. No apparent difference in the clioquinol binding pattern was found among cases of SMON (subacute myelo-optico-neuropathy) with or without a history of clioquinol administration, the patterns being identical to those of healthy subjects.

In order to confirm the binding of clioquinol to albumin, crystalline human albumin was employed in place of serum in a similar experiment (Fig. 2). A single peak of radioactivity corresponding to the position of albumin migration was obtained with clioquinol concentrations ranging from 15 to 60 μg/ml of albumin solution (4.15 g albumin/dl), suggesting that the isolated albumin, which is virtually free of fatty acids (which were measured and found to be

Table 1. Patterns of [^{131}I]clioquinol binding to serum proteins and the serum concentrations of total protein, albumin, bilirubin, NEFA and triglycerides in various diseases and normal controls

Case No.	Disease	Total protein (g/dl)	Albumin (g/dl)	Bilirubin Total (mg/dl)	Bilirubin Direct (mg/dl)	NEFA (mEq/l.)	Triglycerides (mg/dl)	Extra-albumin peak of [^{131}I]-clioquinol Height*	Extra-albumin peak of [^{131}I]-clioquinol Position
1	Metastatic liver cancer	6.4	3.1	26.6	25.8	1.30	257	++	β
2	Liver cirrhosis	7.7	3.2	8.0	5.3	0.80	52	+	$\alpha-\beta$
3	Acute hepatitis	7.2	3.9	35.5	34.6	0.84	406	+	$\alpha-\beta$
4	Primary biliary cirrhosis	8.5	3.8	17.8	13.3	1.45	252	+	β
5	Nephrotic syndrome	4.6	2.1	0.4	0.1	0.38	118	+	β
6	Liver cirrhosis	5.7	3.4	2.7	2.2	0.45	53	$\pm$	$\alpha-\beta$
7	Liver cirrhosis	8.1	3.6	1.1	0.7	0.93	138	$\pm$	$\alpha-\beta$
8	Liver cirrhosis	8.5	4.3	1.6	1.3	0.44	111	$\pm$	$\alpha-\beta$
9	Chronic hepatitis	7.6	4.1	0.8	0.4	0.91	129	$\pm$	$\alpha-\beta$
10	γ-G myeloma	10.9	4.1	0.9	0.2	0.82	94	–	
11	Chronic hepatitis	7.6	3.8	0.5	0.2	0.39	126	–	
12	SMON†, ‡	5.9	3.3	0.8	0.4	0.46	80	–	
13	SMON‡	6.4	4.0	1.1	0.7	0.34	144	–	
14	SMON§	7.4	4.1	0.6	0.1	0.40	170	–	
15	Fatty liver	7.2	4.8	0.7	0.2	0.58	59	–	
16	Fatty liver	7.9	5.4	0.9	0.4	0.76	140	–	
17	Normal control	7.3	5.0	0.9	0.4	0.47	106	–	
18	Normal control	7.4	4.4	0.9	0.4	0.77	119	–	

* Symbols indicate: ++, comparable to the height of albumin-bound radioactivity peak; +, ++ > + > $\pm$; $\pm$, small but recognizable peak; –, no peak, simply a shoulder.

† SMON, subacute myelo-optico-neuropathy.

‡ Without history of clioquinol administration.

§ With history of clioquinol administration.

0.14 mole/mole of albumin), has a greater capacity to bind clioquinol than albumin in serum. Similar results have been reported for sodium sulfobromophthalein binding to albumin [12]. As the relative amount of clioquinol to albumin solution was increased above 100 μg/ml, a tailing of radioactivity became apparent, indicating that a continuous dissociation of the clioquinol-albumin complex is taking place during migration through a clioquinol-free area of agarose. Thus, the radioactivity remaining at the origin represents the closest estimate of the amount of free clioquinol which is in equilibrium with the bound clioquinol in the original clioquinol-albumin mixture. This allows calculation of the concentrations of free and bound clioquinol at equilibrium, respectively, from the fractions of radioactivity remaining at the origin and the remainder on the recorded chart and from the initial clioquinol concentrations in the applied samples. With the molar ratios of bound clioquinol to albumin thus obtained at different concentrations of free clioquinol, the number of binding sites and the apparent binding constant were tentatively estimated by the Scatchard plot to be 0.91 and 5.6×10^4 M^{-1} respectively (Fig. 3).

The presence of clioquinol binding proteins other than albumin was confirmed by separating serum protein fractions by electrophoresis, using sera from Cases 1 and 5 in Table 1, without addition of clioquinol, subjecting them to re-electrophoresis with [^{131}I]clioquinol and demonstrating the migration of radioactivity with each of the α_2-, α_2-β- and β-fractions in addition to the albumin fraction. Since the increased extra-albumin peak of radioactivity was found among patients with higher levels of serum lipids (Table 1), lipoproteins were stained and their mobilities were compared with those of [^{14}C]clioquinol by the radioautographic technique. The migration of the main extra-albumin band of [^{14}C]clioquinol corresponded to that of the β-lipoprotein band. Small amounts of radioactivity were also found in α- and pre-β-lipoprotein bands. In order to confirm the binding of clioquinol to serum lipoproteins, lipoprotein fractions were separated by ultracentrifugation according to Yasugi and Honma [11] and subjected to electrophoresis with [^{14}C]clioquinol. The results obtained with the serum from a patient with nephrotic syndrome (Case 4 in Table 1) are presented in Fig. 4. With an amount of clioquinol in excess of saturating serum albumin, clioquinol binding was observed both in albumin and extra-albumin (corresponding to β-lipoprotein) bands when the whole serum was employed. Electrophoresis of fractionated lipoproteins with clioquinol showed that not only low-density lipoprotein (LDL), which has the electrophoretic mobility of β-lipoprotein, but also very low density lipoprotein (VLDL) and high density lipoprotein (HDL) were capable of binding clioquinol, as revealed by positive radioautography and protein staining in the identical positions. After the removal of the lipoproteins, albumin was the sole clioquinol binding protein among the residual serum proteins. Similar results were obtained with sera from a

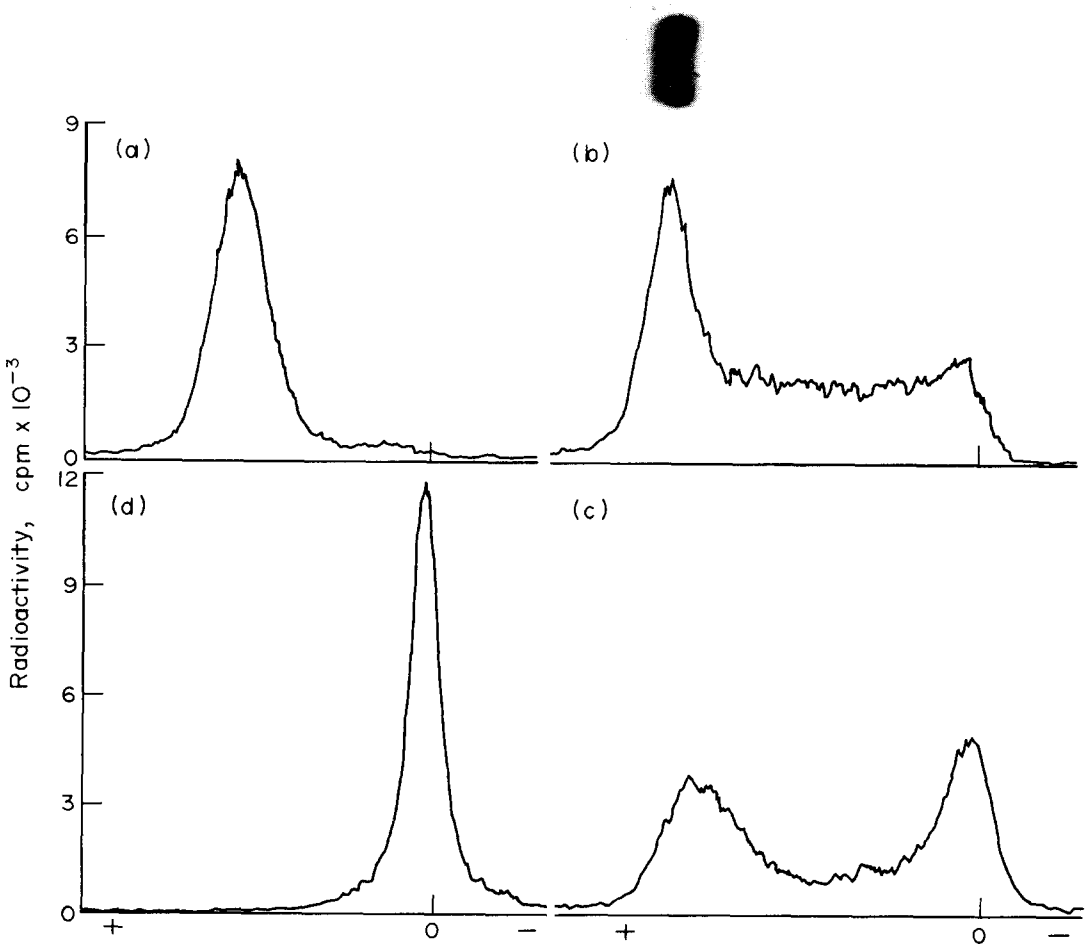

Fig. 2. Electrophoretic patterns of radioactivity with crystalline human albumin. The concentrations of clioquinol in albumin solution (4.50 g albumin/dl) were: (a) 16 μg/ml; (b) 50 μg/ml; (c) 96 μg/ml; and (d) no albumin. The albumin used contained free fatty acids in a molar ratio of 0.14. The photograph at the top shows the pattern of protein staining for b, and 0 denotes the origin.

healthy subject (Case 18) and from a case with primary biliary cirrhosis (Case 4 in Table 1). In a comparison of the free clioquinol radioactivities remaining at the origins among these serum proteins, the capacities of lipoproteins to bind clioquinol in terms of unit protein weight appeared to be greater than that of albumin (cf. the legend to Fig. 4 as to the amounts of applied clioquinol per unit weight of proteins).

DISCUSSION

Studies of clioquinol binding to serum proteins reported by other workers have some limitations in quantitative interpretation of the results because of an interaction of clioquinol with the supporting medium, unless the isolation of bound clioquinol is carried out at very low concentrations of added clioquinol and buffer solution. The difficulties were circumvented in the present studies by employing agarose gel as the electrophoretic medium for separation of free and bound forms of clioquinol. As a result, clioquinol was found to be bound mostly to albumin at the clioquinol concentrations which could be attained in man by oral administration of the drug [14, 15]. This is in agreement with the results obtained in studies *in vivo* in man [2] and other animals [7]. Analysis of the binding data by Scatchard plot revealed that 1 mole of clioquinol is bound per mole of human albumin with a binding constant of $5.6 \times 10^4\ M^{-1}$. The binding of clioquinol to albumin appears to represent a specific type of binding common to that seen among other drugs, i.e. the high affinity of clioquinol to albumin is comparable to that of warfarin [16], phenylbutazone [16] or bilirubin [17]. However, clioquinol binding to albumin is less strong than that of long chain fatty acids [18]. This would explain the extra-albumin binding of clioquinol in cases with increased serum levels of NEFA as resulting from competition between clioquinol and NEFA for albumin and subsequent displacement of clioquinol to lipoproteins.

The binding of clioquinol to lipoproteins was suggested by our previous studies [8] and was demonstrated by Ogata *et al.* [7] with antilipoprotein sera. The present studies further confirmed that lipoproteins are virtually the sole serum components responsible for the extra-albumin binding of clioquinol.

Among the lipoprotein classes, β-lipoprotein or LDL appears to play a major role in constituting the extra-albumin peak, since the β-lipoprotein band and the most intense band of extra-albumin radioactivity were superimposed in the cases studied. However, isolated VLDL and HDL were shown to be as effective as LDL with respect to their binding capacity, suggesting that LDL has a stronger binding affinity to clioquinol than the other lipoproteins. Thus, the small variation in the migration of main extra-albumin peak found among cases studied, i.e. α_2-β region or close to β-globulin, may well be due to the change in electrophoretic mobility of lipoproteins in the absence of added albumin [19].

In contrast to albumin, lipoproteins have weak binding affinities for clioquinol, since a significant binding was observed only after saturating albumin with clioquinol. However, the binding capacities of lipoproteins appear to be greater than those of albumin, because the height of the radioactivity of the extra-albumin peak reached to that of albumin, as the relative amount of clioquinol to serum was increased, in spite of much lower concentrations of serum lipoproteins. This is in accord with the observation made for sodium sulfobromophthalein binding to serum proteins [12]. The binding to lipoproteins might represent a non-specific solubilization of clioquinol into the lipid moiety of lipoproteins, rather than a specific binding to the apolipoproteins, in view of the lipid-soluble nature of clioquinol. The pathophysiological significance of the present observation lies in the fact that, under circumstances where plasma clioquinol concentration exceeds the binding capacity of albumin, clioquinol would readily bind lipoproteins and other lipid-rich constituents of tissues in a manner analogous to the phenomenon observed with bilirubin [20] and sodium sulfobromophthalein [12]. Increased serum concentrations of other albumin binding drugs and endogenous substances, such as NEFA, or hypoalbuminemia of varying causes, would likewise alter the distribution *in vivo* of clioquinol [21]. Alternatively, the binding of clioquinol to lipoproteins may alter the properties of lipoproteins as carriers of physiological substances [22].

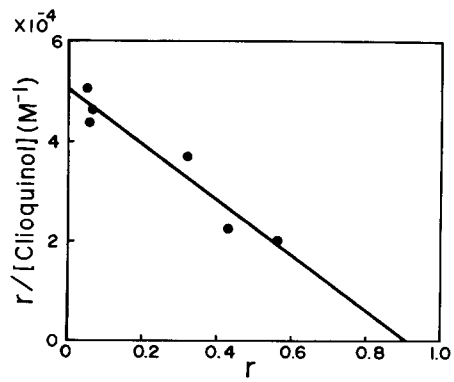

Fig. 3. Scatchard plot of r/[clioquinol] vs r for the binding of clioquinol by albumin at varying concentrations of clioquinol. A molecular weight of 65,000 was assumed for the human albumin. Key: r, the ratio of moles of clioquinol bound to total moles of albumin, and [clioquinol], free clioquinol concentration (moles/liter). The binding data obtained at 20° in barbital buffer, pH 8.6, and ionic strength 0.025, were analyzed by means of the Scatchard equation [13].

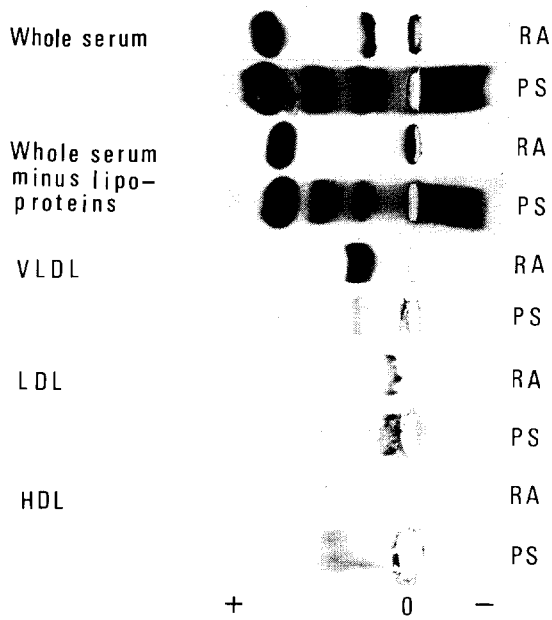

Fig. 4. Binding of clioquinol to serum lipoprotein fractions. Electrophoresis was carried out after mixing [^{14}C]clioquinol with the following: whole serum (16 μg/ml of serum; or 1.2 μg/mg of albumin and 27 μg/mg of protein from lipoproteins); VLDL, very low density lipoprotein (9.9 μg/mg of protein); LDL, low density lipoprotein (3.5 μg/mg of protein); HDL, high density lipoprotein (4.8 μg/mg of protein); and original serum minus lipoproteins (0.46 μg/mg of protein or 0.75 μg/mg of albumin). Electrophoresis of isolated lipoprotein fractions was performed with 0.4% agarose–0.12% agar and others with 1% agarose. Key: RA, radio-autogram; PS, protein staining. Lipoprotein staining gave results similar to those of radioautography except for albumin, which gave very weak lipid staining. The extra-albumin band of radioactivity in the whole serum corresponded to β-lipoprotein on lipoprotein staining with acetylated Sudan Black B. No significant radioactivity was found in α- and pre-β-lipoprotein bands with the whole serum in this case.

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