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Effect of membrane filtration of antimalarial drug solutions on *in vitro* activity against *Plasmodium falciparum**

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Antimalarial activities of chloroquine, mefloquine, amodiaquine, and quinine in vitro *against* Plasmodium falciparum *were diminished as a consequence of membrane filtration. Filtered drug solutions gave ID_{50} values up to 25-fold greater than those of non-filtered (ethanol-sterilized) drug solutions. Loss of activity by filtration was overcome by increasing the drug concentration prior to filtration. Water solutions filtered through Millex-GS filter units consistently showed an absorbance maximum at 277 nm, accompanied by a lesser peak at 225 nm. Water filtrates from Nucleopore and Millex-GV filters showed no absorbance at 277 nm and only slight absorbance was evident for the Gelman filter unit. Activity losses were attributed to extractable contaminating moieties in the membrane filters and/or drug binding to the membrane filters.*

Sterile disposable filter units are widely used for the sterilization of tissue culture media components and drug solutions in various analytical and biological testing procedures. A recent survey of major medical centres in the United States revealed that, although a high proportion (36%) of their clinical laboratories use membrane filtration to sterilize drug solutions for use in quantitative susceptibility tests, none had determined whether membrane filtration had affected drug activity (*1*). While Murray & Niles (*2*) obtained full recovery of antimicrobial activity following filtration of solutions containing 1000 and 100 mg/l of the drug, inhibitory effects of membrane-sterilization of solutions have been reported in mammalian (*3*, *4*), protozoan (*5*), bacterial (*6*), and viral (*7*) culture systems.

Because antimalarial drug solutions are routinely sterilized by membrane filtration for *in vitro* drug testing procedures, we have examined the effects of filtration on the activities of some standard antimalarials against *Plasmodium falciparum* in an *in vitro* susceptibility test system. The results show cause for careful interpretation of drug sensitivity studies.

MATERIALS AND METHODS

Filter membranes

Four different filter membranes were tested: Gelman Acrodisc[a] (esters of cellulose acetate), 25 mm^2 and 0.2 μm pore size; Nucleopore filter disc[b] (polycarbonate), 25 mm^2 and 1.0 μm pore size; Millex-GV filter unit[c] (fluorocarbon), 25 mm^2 and 0.22 μm pore size; and Millex-GS filter unit[c] (mixed esters of cellulose acetate), 25 mm^2 and 0.22 μm pore size.

Drugs

Chloroquine diphosphate, mefloquine hydrochloride, amodiaquine hydrochloride, and quinine sulfate were obtained from the US Army Antimalarial Drug Development Program Inventory in powder form.

Biological activity

The multidrug-resistant Viet Nam Smith strain of *P. falciparum* (*8*) was used as test organism. Cultures were maintained in human A+ red cells in culture medium RPMI 1640[d] supplemented with HEPES buffer and 10% human A+ plasma (*9-11*). Anti-

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[c] Millipore Corp., Bedford, MA, USA.

[d] Gibco Laboratories, Grand Island, NY, USA.

malarial activity was measured as previously described (*9*, *10*) using 96-well microtitration plates. Inhibition of incorporation of [G-^{3}H]-hypoxanthine[e] by the parasites served as an index of antimalarial activity. The drugs were initially dissolved in 70% ethanol to a concentration of 1000 mg/l and subsequent dilutions were made with the culture medium. Membrane filtration of the drug solutions is not required for sterilization in this test system.

To determine the effect of filtration on antimalarial activity, stock solutions of the drugs were prepared in 70% ethanol to an initial concentration of 2000 mg/l and then diluted further in distilled water to a final concentration of 2 mg/l. Ten millilitres of the 2 mg/l drug solution were then filtered through each of four filter types. Each filtrate was then serially diluted 2-fold with culture medium in the microtitration plates.

The data were analysed by nonlinear regression analysis (*9*, *10*) to obtain the 50% inhibitory dose (ID_{50}), i.e., the drug concentration corresponding to 50% inhibition of the uptake of radiolabelled hypoxanthine by the parasites.

Ultraviolet absorbance spectrophotometry of water filtrates

Nucleopore membranes were not housed in disposable units but were placed into sterilized stainless steel filter housings for fitting to syringes. The membranes, handled with stainless steel forceps at all times, were sterilized by autoclave (120 °C, 1.4 kg/cm^2 for 15 minutes) and dried at 25 °C prior to use. Each individual membrane was fitted to a 10-ml plastic syringe, and 3 ml of sterile, distilled and deionized water were passed through the filter unit. The filtrate was immediately dispensed into a quartz cuvette and an absorbance profile was obtained for the 200–350 nm range in a Varian-Cary model 219 spectrophotometer.[f] Possible contributions by other items of the apparatus were checked by repetition of the technique with the membranes omitted.

Spectrophotometric measurement of drugs and drug filtrates

Drug losses due to filtration were quantified as follows. Chloroquine, mefloquine, amodiaquine and quinine were made at a concentration of 2000 mg/l in 70% ethanol. Subsequent dilutions were made with sterile distilled and deionized water to a final concentration of 2.0 mg/l; 10.0 ml of each solution were passed through each filter type. Absorbancy before and after filtration was measured at the optimal wavelengths for each drug (chloroquine, 343 nm; mefloquine, 310 nm; amodiaquine, 340 nm; and quinine, 330 nm). The percentage drug loss due to filtration was calcuated using the equation:

$$\% \text{ drug loss} = 100 - \left[\frac{\text{absorbance after filtration}}{\text{absorbance before filtration}} \times 100 \right]$$

Chloroquine in solution is known to obey Beer's law (*12*). We also observed a linear relationship between UV light absorbance and drug concentration for mefloquine, amodiaquine, and quinine at their respective wavelengths of absorbance measurement.

RESULTS

The biological activity of the antimalarials after their filtration through the individual filter types is summarized in Table 1. Decreased antimalarial activity was most evident with amodiaquine regardless of the filter types used, whereas quinine had the least deviation from the ethanol-sterilized standard activity. All compounds showed marked binding to the Millex-GV membranes (67–98%).

The results on the antimalarial activities of chloroquine and mefloquine solutions filtered through Millex-GV filter units at two different concentrations are given in Table 2. Solutions filtered at a concentration of 2000 mg/l gave ID_{50} values comparable to those of nonfiltered drug solutions whereas those

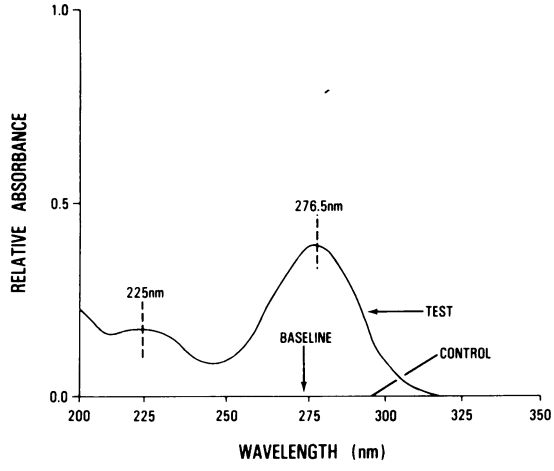

Fig. 1. UV spectra: TEST, water passed through a Millex-GS filter unit; CONTROL, water passed through a plastic syringe with membrane omitted; BASELINE, untreated water.

[e] New England Nuclear, Boston, MA, USA.

[f] Varian Associates Inc., Palo Alto, CA, USA.

Table 1. ID_{50} values of non-filtered and filtered solutions of chloroquine, mefloquine, amodiaquine and quinine against *P. falciparum*

Drug filter	% drug bound[b]	ID_{50} (mg/l)[a] Observed[c]	Corrected[c]	Fold increase
Chloroquine:				
Ethanol	–	54.0	54.0 (1.7)	1.0
Nucleopore	2	> 145.0	> 196.0	> 2.0
Gelman	5	98.0	95.0 (8.3)	1.7
Millex-GV	75	> 200.0	> 50.0	> 0.92
Millex-GS_1[d]	22	> 150.0	> 176.0	> 2.4
Millex-GS_2[d]	22	120.0	110.0 (3.9)	2.0
Mefloquine:				
Ethanol	–	1.5	1.5 (0.27)	1.0
Nucleopore	0	4.2	4.2 (0.51)	2.8
Gelman	11	4.0	3.7 (0.19)	2.5
Millex-GV	87	38.0	4.6 (0.42)	3.1
Millex-GS_1	ND[f]	4.0	ND	–
Millex-GS_2	ND[f]	3.7	ND	–
Amodiaquine:				
Ethanol	–	7.6	7.6 (1.68)	1.0
Nucleopore	2	> 56.0	> 49.0	> 3.9
Gelman	7	> 56.0	> 47.0	> 6.1
Millex-GV	98	> 56.0	–[e]	–
Millex-GS_1	12	> 56.0	> 44.0	> 5.8
Millex-GS_2	12	> 56.0	> 44.0	> 2.0
Quinine:				
Ethanol	–	54.0	54.0 (5.5)	1.0
Nucleopore	0	72.0	72.0 (0.69)	1.3
Gelman	4	120.0	113.0 (4.1)	2.0
Millex-GV	67	140.0	59.0 (3.3)	1.1
Millex-GS_1	10	120.0	106.0 (2.9)	1.9
Millex-GS_2	10	58.0	55.0 (5.6)	1.0

[a] Values of ID_{50} are the average of four determinations performed on separate occasions. Numbers in parentheses are the standard error of the nonlinear regression analysis (*17*).

[b] For calculation, see pp. 439–440 (Materials and Methods).

[c] Observed ID_{50} was adjusted by multiplying the percentage drug bound by the observed ID_{50} and subtracting the product from the observed ID_{50} to give the corrected value.

[d] Millex-GS_1 was taken from a lot known to contain a relatively high concentration of contamination as determined by filtrate absorbance at 277 nm (0.5, see Fig. 1). Millex-GS_2 was taken from a lot known to contain a relatively low concentration of contamination (0.05, see Fig. 1).

[e] Out of the test range, owing to the high degree of drug binding to the filter membrane.

[f] Owing to spectral interference from the Millex-GS contaminant (A_{277}), spectrophotometric determination of mefloquine (A_{310}) loss to these filters was not done. Thus, no data may be given for the corrected ID_{50} and fold increase for this drug passed through these filters.

filtered at 10 mg/l showed appreciable reductions in activity.

Evidence that a contaminating moiety is extracted when water solutions are filtered through Millex-GS filter units is shown in Fig. 1. In several filtrates of distilled water (or physiological saline, data not shown) an absorbance maximum at 277 nm was always evident, accompanied by a lesser peak at 225 nm.

Absorbances at 277 nm in water filtrates from several lots of Millipore's GS type membranes (filter discs and filter units) as well as from several samples of Gelman, Nucleopore and Millex-GV filters are summarized in Table 1. No absorbance was contributed by the Nucleopore and Millex-GV filters, and only slight absorbance was evident in the Gelman filter unit filtrates.

Table 2. Antimalarial activity of Millex-GV filtered and non-filtered solutions of chloroquine and mefloquine solutions against *P. falciparum*

Drug	Filtered solutions (mg/l)	ID_{50} (μg/l)
Chloroquine	not filtered	51.0 (3.7)[a]
"	10	124.0 (4.8)
"	2000	59.0 (5.3)
Mefloquine	not filtered	1.7 (0.36)
"	10	28.0 (2.7)
"	2000	1.0 (0.31)

[a] Values are the average of duplicate determinations performed on two separate occasions. Numbers in parentheses are the standard error of the nonlinear regression analysis (based on information in a pamphlet describing nonlinear regression analyses and computer programming by D. Rodbard, Biomedical Computing Technology Information Center, Nashville, Tennessee, 1980).

DISCUSSION

The effects of membrane filtration on antimalarial drug solutions and their subsequent activities *in vitro* have been shown in this study. Drug solutions, when filtered at low concentrations through different types of membrane filter units, may lose their activity *in vitro* against *Plasmodium falciparum*. We attribute the losses in antimalarial activity to at least two factors: (*a*) drug binding to membrane filters and (*b*) materials extractable from the membranes into the drug solution filtrates.

The highest percentage of drug binding was evidenced with the Millex-GV filter unit, ranging from 67% for quinine to 98% for amodiaquine, which was reflected in the observed ID_{50} values for the antimalarials tested (see Table 1). In addition, the data from Table 2 indicate that filtration of chloroquine or mefloquine solutions at high concentration (2000 mg/l) through Millex-GV filters did not affect drug activity; filtration at low concentration (10 mg/l) affected activity appreciably. This suggests that solutions at high concentration may have overwhelmed the capacity of the membrane filters to bind the drug, which may have otherwise influenced the antimalarial activity.

Binding of the drug to the filter, however, does not account for all loss of activity. This is most evident in the results obtained with the Nucleopore and Gelman filters (Table 1). Although these filters showed $\leqslant 11\%$ binding of the drug, the diminution in drug activity was consistently in excess of that expected at such levels of drug-filter binding. Most membrane filters contain nonionic detergents, such as Triton X-100 (which may comprise 2–3% of their dry weight) to improve fluid flow (*13*). These detergents or other materials present in the membrane may be extracted during the filtration process and appear in the filtrates. Technical information provided by manufacturers do not mention this possibility. An absorbance maximum in the ultraviolet light wavelength region of 275 to 277 nm was found by Rosenbluth & Cripps (*14*) in both water filtrates from cellulose-based filter media and in a nonfiltered aqueous solution of isooctyl-phenoxy-polyethoxy-ethanol (IPPE, Triton X-100). By measuring the ultraviolet light absorbance of water filtrates from both cellulose and polycarbonate filter media, Cooney (*15*) detected appreciable amounts of contaminant(s). In our study the presence of a contaminant with an absorbance maximum at 277 nm was demonstrated in water filtrates of Millipore-GS type filters (Fig. 1, Table 3); absorbancy at this wavelength was not detected in filtrates from fluorocarbon or polycarbonate membranes. We suggest that materials extracted from these filter units into the drug filtrates, which are not necessarily detectable by ultraviolet spectroscopy, may antagonize *in vitro* the antiplasmodial activity of the standard drugs tested. It may not be stated with certainty that the contaminating moieties characterized by UV light absorbance at 277 nm were directly responsible for activity diminution.

In summary, our study indicates that filtration of antimalarial drug solutions at low concentrations

Table 3. Absorbance at 277 nm of distilled water filtrates; a volume of 3.0 ml was passed through each filter unit

Filter type		Absorbance at 277 nm
Millipore-GS	C1H11116[a]	0.036 (0.0025)[b]
"	C1H09169	0.060 (0.005)
"	C8P80362	0.067 (0.003)
"	C8N75415	0.229 (0.015)
"	C8N78264	0.373 (0.030)
Gelman Acrodisc[c]		0.017 (0.004)
Nucleopore disc[c]		0.000 –
Millex-GV unit[c]		0.000 –

[a] Code following Millipore-GS tests denote manufacturer's lot numbers.

[b] Numbers in parentheses are the standard error of 5 to 10 determinations.

[c] At least three different manufacturer's lot numbers were sampled and 3 to 5 filters from each lot were examined.

results in losses of drug activity as a consequence of chemical impurities and/or drug loss from solution during filtration. To prevent erroneous reporting of drug susceptibility studies, we strongly recommend that drug solutions be filtered at high concentration followed by aseptic serial dilution of the filtrate. This practice would minimize the percentage drug loss to the filter apparatus and dilute out any extracted contaminants if present. Another possibility may be the initial dissolution of the drug in ethanol with subsequent serial dilutions performed aseptically. A similar statement of caution has been expressed by Geary et al. (*16*) concerning chloroquine binding to glass and cellulose acetate filters.

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RÉSUMÉ

MODIFICATION DE L'ACTIVITÉ *IN VITRO* DES ANTIPALUDÉENS FILTRÉS SUR MEMBRANE VIS-À-VIS DE *PLASMODIUM FALCIPARUM*

Les unités de filtration stérile à usage unique sont largement utilisées dans différentes épreuves biologiques et méthodes analytiques pour la stérilisation des différents composants des milieux de culture tissulaire et celle des solutions de médicaments. De nombreux laboratoires cliniques ont recours à la filtration sur membrane pour stériliser les solutions de médicaments destinées à des épreuves quantitatives de sensibilité; aucun cependant n'a établi si ce mode de filtration avait modifié l'activité du médicament. On a déjà signalé un effet inhibiteur de ce procédé de stérilisation des solutions sur les cultures de cellules mammaliennes et celles de protozoaires, de bactéries et de virus. Vu que les solutions d'antipaludéens sont couramment stérilisées par filtration sur membrane en vue des essais *in vitro* des médicaments, l'effet de la filtration des antipaludéens à usage clinique et leur activité sur *Plasmodium falciparum* après filtration ont été étudiés dans une épreuve de sensibilité *in vitro*.

Quatre membranes filtrantes ont été mises à l'essai: Gelman Acrodisc (desacétate de cellulose), disque filtrant Nucléopore (polycarbonate), Millex-GV (fluorocarbone) et Millex-GS (mélange d'esters cellulosiques). La chloroquine (sous forme de diphosphate), la méfloquine, l'amodiaquine (chlorhydrate) et la quinine (sulfate) ont été obtenues en poudre auprès de l'U.S. Army Antimalarial Drug Development Program. La souche Smith de *P. falciparum* a été utilisée pour mesurer l'activité biologique. Les cultures ont été entretenues au moyen de la méthode de Trager-Jensen. Comme indicateur de l'activité antipaludique, on a utilisé l'inhibition de l'incorporation d'hypoxanthine tritiée [G-^{3}H] par le parasite; dans cette méthode de mesure, les solutions de produits n'ont pas besoin d'être filtrées sur membrane. Afin de préciser l'influence de la filtration sur l'activité antipaludique, 10 ml d'une solution de médicament à 2 mg/l ont été filtrés sur chacune des unités de filtration. Les résultats ont été analysés par régression non linéaire pour obtenir la DI_{50}. De l'eau distillée a été filtrée et les spectres d'absorption ont été établis entre 200 et 350 nm. Les solutions de médicaments (à 2 mg/l) ont été passées au spectrophotomètre avant et après filtration; la mesure a été effectuée pour chacun des médicaments à la longueur d'onde optimale soit 343 nm. Les solutions de médicaments (à 2 mg/l) ont été passées au spectrophotomètre avant et après filtration; la mesure a été effectuée pour chacun des médicaments à la longueur d'onde optimale soit 343 nm pour la chloroquine, 310 nm pour la méfloquine, 340 nm pour l'amodiaquine et 330 nm pour la quinine. La perte de produit due à la filtration a ensuite été calculée en pourcentage.

Une baisse de l'activité antipaludique est apparue après filtration sur certains des filtres. Des DI_{50} se sont parfois montrées 25 fois plus élevées qu'avec les témoins non filtrés. Tous les composés se sont abondamment fixés sur les membranes Millex-GV (67 à 98%). Les solutions filtrées de chloroquine et de méfloquine à 2000 mg/l ont donné une DI_{50} comparable à celle des solutions non filtrées; par contre, une perte d'activité appréciable a été constatée avec les solutions à 10 mg/l filtrées. En ce qui concerne l'eau distillée filtrée sur unité Millex-GS, on a régulièrement obtenu un pic d'absorption à 277 nm accompagné d'un pic secondaire à 225 nm; les filtres Nucléopore et Millex-GV n'ont donné lieu à aucune absorption à 277 nm, tandis qu'elle était très faible avec l'unité de filtration Gelman. La perte d'activité a donc été attribuée à des fractions extractibles correspondant à des impuretés contenues dans les membranes filtrantes et/ou à la fixation du médicament sur les membranes. Les résultats obtenus montrent en conséquence qu'il y a lieu d'interpréter avec circonspection les études de sensibilité *in vitro*. De même, il serait souhaitable que les résultats de l'étude *in vitro* de la pharmacorésistance des plasmodies soient accompagnés de la description des mesures prises pour que la filtration sur membrane des solutions d'antipaludéens ne contribue pas à réduire leur activité.

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