A NEW VIAL MICROASSAY TO SCREEN ANTIPROTOZOAL DRUGS

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Summary

A new microassay to screen compounds having activity against *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis* is described. The procedure is performed in 1 mL capacity glass screw-capped vials, which have an external cylindrical shape and a conical interior. Vials were filled with 950 μL of a trophozoite suspension (2×10^4 to 2×10^5/mL) and 50 μL of the test substance at a final concentration of 1.6–50 μg/mL. Emetine, metronidazole, tinidazole, secnidazole, ornidazole and dimetridazole were used as standards. The preparations were incubated at 36 °C for 24–48 h and the concentration of each drug producing 50% growth inhibition was calculated. All results obtained with this vial micromethod were within the ranges reported by other authors in each particular case. The vial assay method combines the advantages of a microassay, requiring small quantities of test substances and with the functionality of assays in culture tubes.

Running title: Antiprotozoal vial microassay

Key words: *Entamoeba histolytica*, *Trichomonas vaginalis*, *Giardia lamblia*, *antiparasitic drugs, micromethod, vial micromethod.*

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Introduction

Human diseases caused by parasitic protozoa such as *Entamoeba histolytica, Trichomonas vaginalis* and *Giardia lamblia* occur with high prevalence worldwide [1, 2]. Among the human parasitoses, amebiasis is the second greatest cause of death globally, malaria being the first. All of these parasitosis are widely distributed in industrialized and developing countries, but affect mainly developing countries [3-5].

Amebiasis is characterized by destruction of the organs and tissues invaded, and its major clinical manifestations are dysentery and hepatic abscesses [1, 6].

Trichomoniasis is a common cause of vaginitis, urethritis and prostatitis [4] and has been linked to sterility problems, low birth weights and preterm delivery [7]. The most frequent manifestations of *G. lamblia* infection are in the human and animal small intestine [8, 9] and it is a common cause of urticaria, angioedema [10, 11] and atopic dermatitis [12, 13].

Metronidazole is one of the most efficacious medications for the treatment of amebiasis, trichomoniasis and giardiasis [14]. Nevertheless, 2.5% to 5% of the causal agents of these diseases, *E. histolytica, T. vaginalis* and *G. lamblia* respectively, display some level of resistance to metronidazole [15, 16]. Tinidazole has been shown to be a good alternative [17, 18, 19].

Infections by the above protozoa species have been treated with metronidazole and its derivatives such as tinidazole, secnidazole and ornidazole [15, 20-22]. Similarly, emetine has been successfully used to treat amebiasis, and albendazole and mebendazole used to cure giardiasis [23]. However, the armamentarium against these parasitic protozoa is relatively small and the possibility of resistant strains represents a real risk. Thus, the development of new medications is a very advisable task.

A frequent approach to starting the development of new medications against an infectious or parasitic agent is to identify natural, semisynthetic or synthetic products that are active in vitro against the species of interest. In these cases, reliable bioassays constitute an essential tool and constant

improvement of these assays is required.

The use of test tubes to perform standard bioassays or microtiter plates to carry out microassays are the most frequent methods used in screening new antiprotozoa products. Test tubes are easy to handle and monitor. Nevertheless, when the test material is available in only small quantities, this practice is unviable because 6 to 15 mL of test medium and a proportional mass of the screened product are needed to fill a test tube. On the other hand, a microassay needs strikingly less quantities of test medium and the product for testing. The most frequently used assays are based on 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan reduction [24-28]. Nevertheless, this method requires several steps for washing and dissolution—the formazan produced in the cells must be extracted with isopropanol–HCl and the resultant absorbance measured at 570 nm. Another micromethod is based in [^{3}H]-thymidine uptake to estimate the 50% inhibitory dose [29]; however, the paraphernalia associated with the use of radioactive compounds is expensive and its availability complicated. Thus, we present here a new microassay, which is a methodological alternative that incorporates the advantages of test tubes and microplates, allowing high reproducibility to be obtained with easy handling and monitoring.

Material and Methods

Parasite cultures

E. histolytica strain HM-1:IMSS, *T. vaginalis* strain GT-13 and *G. lamblia* strain 0989:IMSS were used in this study. *E. histolytica* and *T. vaginalis* were grown in a medium named PEHPS, which is and a acronym of its main components written in Spanish, casein peptone, liver and pancreas extract pancreas and bovine serum [30] and *G. lamblia* in TYI-S-33 supplemented with bile [31]. All three species were subcultured three times each week. Parasites used in the assays to determine drug susceptibility were harvested when cultures had reached the middle of their respective logarithmic growth phase.

Stock solutions

The concentration of all standard drugs was adjusted at 1 mg/mL. Metronidazole, tinidazole, secnidazole, ornidazole, dimetridazole and emetine were dissolved in double distilled water, and albendazole and mebendazole dissolved in dimethyl sulfoxide (DMSO). All stock solutions were stored at –20 °C until used. Immediately before the assays, serial two-fold dilutions of the stock solutions were made in basal PEHP medium (without serum). Fifty microliters of each solution was put into 1 mL glass screw-capped cylindrical vials with a conical interior (vial micro storage Cat. No. 2070-00001, Bellco Biotechnology, Bellco Glass Inc., Vineland, NJ, USA). All vials were filled with 950 µl of a freshly prepared parasite suspension in PEHP medium plus 10% bovine serum, with *E. histolytica*, *T. vaginalis* and *G. lamblia* at concentrations of 2×10^4, 1×10^5 and 2×10^5 trophozoites/mL respectively. All vials were incubated at 36 °C. Those vials containing *E. histolytica* were incubated for 72 h and those with *G. lamblia* or *T. vaginalis* for 24 h. The vials were then chilled in ice water for 20 min, and the number of trophozoites per milliliter in each tube was counted using a hemocytometer. The percentage of growth inhibition with respect to untreated controls was then determined. The 50% inhibitory concentration (IC_{50}) of each drug was calculated by probit analysis [32]. Each drug was assayed in triplicate three times with each protozoan species and the mean and 95% confidence limits calculated.

Chemicals

All chemicals were reagent grade. DMSO and the standard drugs were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except for secnidazole, which was kindly donated by Rhône-Poulenc Rorer Laboratories (México D. F., Mexico). The other chemicals used in this study were purchased from J. T. Baker (Xalostoc, Edo. de Mexico, Mexico). Sterile bovine serum and PEHPS medium were prepared in our laboratory as described elsewhere [30].

Results and Conclusions

Tables 1 to 3 show the results obtained with the vial micromethod and values obtained by other methods, as taken from the literature. In all cases, the IC_{50} values calculated from the vial micromethod were within the reported ranges for the respective parasite species and antiprotozoal drugs. The IC_{50}s of secnidazole and dimetridazole against *E. histolytica* have been reported by Mata et al. [33]. The IC_{50}s of secnidazole and ornidazole against *G. lamblia* have also been determined by one group [25], and those of tinidazole and secnidazole against *T. vaginalis* by two groups [21, 34]. To our knowledge, no reports of the activity of dimetridazole against *G. lamblia* or ornidazole and dimetridazole against *T. vaginalis* have been published.

We have described here a new method that combines the advantages of most currently used micromethods and classical methods, and have determined it to be equally as reliable. In addition, the vials used are reusable because they are made of glass, leading to reduced pollution.

Table 1. In vitro drug susceptibility assays for *E. histolytica*.

	IC_{50} (95% confidence limits) [µg/ml])		
Drug	Vial-micromethod	Other methods	References
Emetine	1.630 (1.520 – 1.740)	0.082 – 1.60	32, 35-37.
Metronidazole	0.711 (0.618 – 0.803)	0.027 – 12.5	32, 35, 37, 38.
Tinidazole	0.160 (0.140 – 0.180)	0.032 – 0.180	32, 35.
Secnidazole	0.161 (0.106 – 0.215)	0.007 – 0.19	33,46.
Ornidazole	0.120 (0.107 – 0.133)	0.032 – 0.125	35, 39.
Dimetridazole	0.182 (0.158 – 0.205)	0.08 -0.20)	33,46.

Table 2. In vitro drug susceptibility assays for *G. lamblia*.

	IC_{50} (95% confidence limits) [μg/ml])		
Drug	Vial-micromethod	Other methods	References
Metronidazole	0.512 (0.445 – 0.579)	0.21 – 1.0	20, 39-42.
Tinidazole	0.132 (0.116 – 0.148)	0.10 – 0.5	41.
Secnidazole	0.140 (0.122 – 0.158)	0.62 – 0.76	26.
Ornidazole	0.165 (0.143 – 0.187)	0.12 – 0.150	26.
Dimetridazole	0.163 (0.141 – 0.184)	Not reported	
Albendazole	0.026 (0.016 – 0.033)	0.01- 0.04	26, 27, 41, 45.
Mebendazole	0.024 (0.015 – 0.032)	0.06 – 0.50	26, 40, 41, 45.

Table 3. In vitro drug susceptibility assays for *T. vaginalis*.

	IC_{50} (95% confidence limits) [μg/ml])		
Drug	Micro method vials	Other methods	References
Metronidazole	1.040 (0.882 – 1.198)	0.90 -6.80	43, 44.
Tinidazole	0.270 (0.170 – 0.370)	0.10 – 0.6	12.
Secnidazole	0.153 (0.100 – 0.206)	0.50 - 2.00	16.
Ornidazole	0.170 (0.150 – 0.190)	Not reported	
Dimetridazole	0.157 (0.137 – 0.177)	Not reported	

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