Journal of Chromatography, 572 (1991) 291–301
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6064

Isolation of microfilariae from blood by gravitational field-flow fractionation[a]

ANNABELLE MERINO

Laboratoire de Chimie Analytique et d'Electrochimie Organiques, Université de Paris-Sud, Centre Pharmaceutique de Châtenay-Malabry, Rue J. B. Clement, F 92296 Châtenay-Malabry (France)

CHRISTIAN BORIES and JEAN-CHARLES GANTIER

Laboratoire de Parasitologie, Université de Paris-Sud, Centre Pharmaceutique de Châtenay-Malabry, Rue J. B. Clement, F 92296 Châtenay-Malabry (France)

and

PHILIPPE J. P. CARDOT*

Laboratoire de Chimie Analytique et d'Electrochimie Organiques, Université de Paris-Sud, Centre Pharmaceutique de Châtenay-Malabry, Rue J. B. Clement, F 92296 Châtenay-Malabry (France)

(First received April 2nd, 1991; revised manuscript received June 26th, 1991)

ABSTRACT

Over 100 million persons suffer from diseases caused by filariae infestation, and one billion are at risk. A simple isolation method for both analytical and preparative separation is presented. Based on the simplest field-flow fractionation technique, the gravitational one, effective isolation of microfilariae is achieved. Microfilariae are eluted in the void volume of the channel without pollution by red blood cells. The red blood cell elution peak shows a total absence of microfilariae, as demonstrated after fraction collection and microscopic investigation. The elution mode of microfilariae and red blood cells appears to be a steric one, as confirmed by a reinjection experiment. The simplicity, low cost and the relatively short time required for this separation (10 min) indicate that gravitational field-flow fractionation could become a new separation tool for screening of microfilariae. With both live and dead microfilariae, the high recovery (66–80%) allows preparative fractionation for diagnostic purposes or fundamental research.

INTRODUCTION

In 1987, the World Health Organization (WHO) estimated that about 100 million persons are infected with lymphatic filariae (*Wuchereria bancrofti, Brugia malayi*), with dermic filariae (*Loa loa*) and with peritoneal filariae (*Mansonella perstans* and *Mansonella ozzardi*). Over one billion people are at risk of infection in endemic areas, where blood-sucking flying arthropods transmit these diseases [1]. The parasite larval stage, named microfilaria (mf), is blood-dwelling, and the

[a] Presented at the *Second International Symposium on Field-Flow Fractionation, Febr. 4–6, 1991, University Park Hotel, Salt Lake City, UT* (Poster).

0378-4347/91/$03.50

laboratory diagnosis of these tropical endemic diseases for case control or epidemiological studies is mainly achieved through detection and identification of blood microfilariae. Staining of 20–40 μl of blood on a thick smear is the most commonly used method. The sensitivity of this method can be increased by saponin lysis of red blood cells [2] and membrane filtration of blood [3]. Both of these techniques take 30–60 min. Field-flow fractionation (FFF), which has been in discrete and continuous development for more than twenty years, is a "one-phase chromatography technique" particularly well suited for the separation of macromolecules and particles. The simplest subtechnique, gravitational FFF (GFFF), which associates the force created by the gravitational field to a perpendicular parabolic flow profile established in a ribbon-like channel, is particularly well suited for particles with a diameter varying from 1 to 100 μm. Red blood cells (RBC) with an average radius of approximately 3–5 μm (80–150 μm^3) are selectively retained, as demonstrated by Caldwell *et al.* [4]. Since mf have a much greater volume (2200 μm^3, *i.e.* 200 μm length, 1.75 μm radius), this huge size and volume difference can be used to separate mf from RBC using GFFF. According to the probable steric elution mechanism [5] of these live particles, the expected elution order is mf followed by RBC.

EXPERIMENTAL

For the experiments presented in this report, mf from the filarial worm *Molinema dessetae*, which infects the rodent *Proechimys oris rodent* were chosen. Adult worms live in the peritoneal cavity of the rodent and the mf are blood-dwelling. This model is used for chemotherapeutic and host–parasite relationship studies [6,7]. Briefly, four- to six-week-old laboratory-bred rodents were infected subcutaneously with 200 infective larvae obtained from mosquitoes (*Aedes aegypti*) previously infected three weeks before on a microfilaremic rodent. After an average incubation period of 90 days, microfilaremia increased progressively and within 26 weeks reached a level of 25–300 mf per 10 μl of blood. Blood samples containing live mf are obtained by puncture of the retro-orbicular sinus of the rodent. A 0.4–ml aliquot of blood was immediately mixed with 0.1 ml of 5% citrate solution in distilled water. The mf remained alive for 24 h at room temperature. The mf were microscopically counted in three Giemsa-stained 10-μl-thick smears for each sample. For experiments with dead parasites, mf were killed by gently heating (45°C) for a few minutes. Before injection, blood samples were diluted with the same volume of physiological saline. A 50-μl Unimetric syringe was used to inject the diluted sample. A control experiment was performed to verify that no difference occurred in the mf count by the thick-smear technique using either the Gilson P20 or the Unimetric syringe (internal diameter of the needle: 130 μm, Gilson, Villiers-le-Bel, France). The mf characteristics were as follows: the mf were unsheathed, the overall length of live mf in extension being 180–220 μm and their width 3–4 μm. On a microscope slide, they move with fast,

jerky, coiling movements without displacement of the erythrocytes. With an mf volume of 1900 μm^3 and a rodent RBC volume of 80 μm^3, the volume ratio of mf to RBC is 24. The microfilariae density is higher than that of the nucleated cells of the blood and lower than that of the RBC, as demonstrated in a sucrose gradient density centrifugation [8]. It should be pointed out here that live and dead mf do not present the same form on microscopic observation: dead mf are rod-shaped, while live ones are coiled.

A GFFF apparatus analogous to the one already described by Giddings *et al.* [9], with glass plates coated with a biocompatible silicone (Silbione, Rhône Poulenc, Paris, France), was set up. The channel was 86 cm long, 2 cm wide and 0.175 mm thick with a dead volume of 3.11 ml including the connection tubes and the detection volume. The detection was performed with a UV–VIS chromatographic spectrophotometer (Shimadzu SPD 6A at 313 nm). The carrier phase was an isotonic sodium chloride solution (0.15 mol/l). A 50-μl aliquot of a two-fold dilution of blood sample was injected with a 10-min relaxation time into the channel. The relaxation injection protocol was simple: the carrier phase flow was stopped and the particles were injected into the channel; 10 min later the flow was set at a velocity of 0.48 cm/s (Waters 6000 A chromatographic pump). Under these conditions, the rodent RBC population had a retention factor of 0.43 (ratio of elution volume of unretained species to elution volume of RBC). The fractions were collected manually under time control. The collected fractions containing the particles diluted in the carrier phase were checked and directly evaluated by optical microscopy (Leitz, Wetzlar, Germany) with a 400-fold magnification for the RBC evaluation and a 100-fold magnification for mf. The quantitation of the collected fractions was performed on a 50-μl Nageotte (Preciss, Paris, France) cell-counting device.

RESULTS

Elution profiles of blood samples with and without parasites

Two blood samples were eluted under the same conditions. Both fractograms recorded at the same attenuation for the first peak are shown in Fig. 1. Fig. 1A presents the normal fractogram and Fig. 1B shows the parasite-containing blood. On the fractogram of Fig. 1A, the peak of the greatest retention volume (peak C) is the RBC peak, and the peak of a retention volume equal to the void volume of the channel (peak B) represents the elution of diffusing species not affected by the external field, *e.g.* proteins. The first peak (peak A) with a retention volume lower than the void volume of the channel represents species unaffected by the external field and with a low diffusion coefficient (cell membranes, cellular organelles). In Fig. 1B, a similar profile is observed; only the intensities of the first two peaks have changed. In the case of parasite-containing samples it is also possible to observe spikes on the second peak which may be characteristic of the detection of particles. On the basis of numerous elutions of blood samples, it has been ob-

served that the intensities of the first two peaks are not reproducible and cannot be attributed to the presence of mf. As there is no specific mf peak in the fractogram, the observation of the presence of mf in the eluate must be performed by optical microscopy. The elution fractions which were systematically observed under the microscope are shown in Fig. 1B. The presence of mf in fraction I is observed at a retention volume equal to the void volume of the channel. Fraction II shows the expected presence of RBC and the total absence of mf, as shown in Fig. 2.

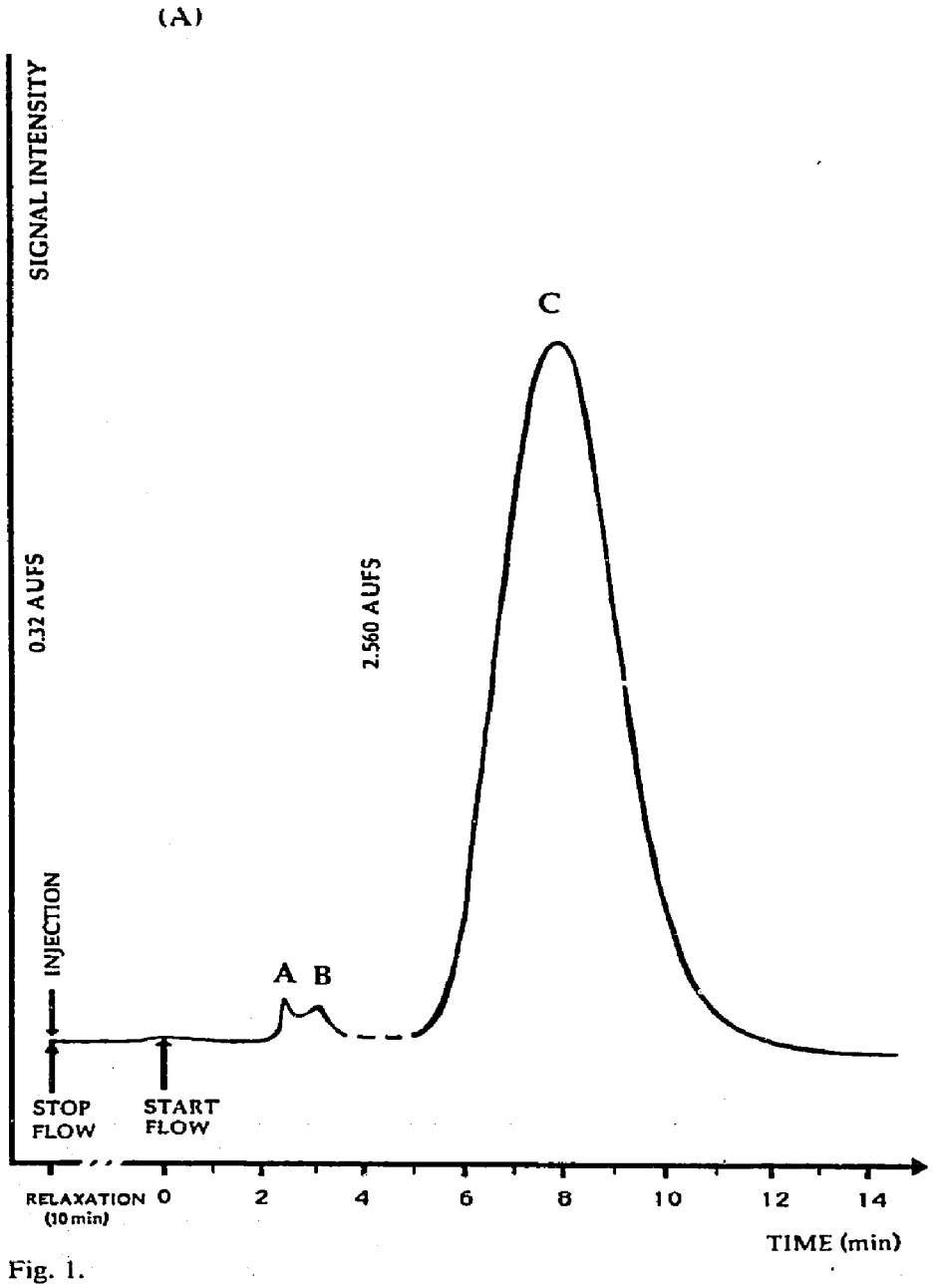

Fig. 1.

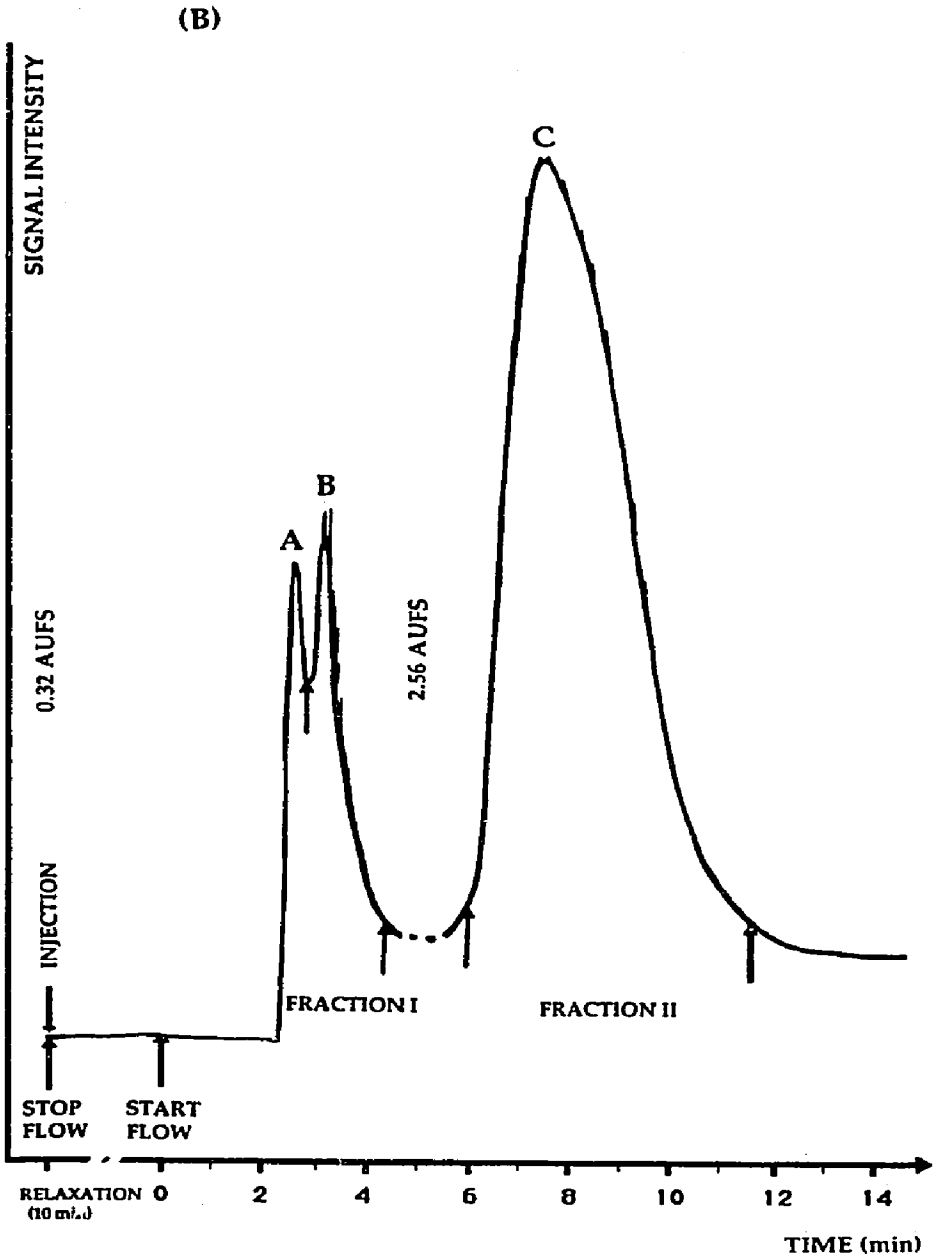

Fig. 1. Gravitational field-flow fractionation elution of rodent blood sample. (A) A 50-μl blood sample dilution (1:2) free of microfilariae. (B) A 50-μl blood sample dilution containing live microfilariae. A 25-μl aliquot of a blood sample was diluted with an equal volume of citrated isotonic solution and injected into the GFFF channel. A 10-min "relaxation" injection protocol was used. Channel characteristics: 86 cm × 2 cm × 0.175 mm; total dead volume including connection and detection device: 3.11 ml. Carrier phase composition: isotonic sodium chloride solution (0.15 mol/l). Flow-rate: 0.48 cm/s. Detection conditions: photometric detection at 313 nm. Fractions I and II represent the elution volumes collected.

Retention characteristics of microfilariae

A series of parasited-containing RBC was performed, and fractions containing peaks A and B were collected and pooled in order to obtain a pure suspension of

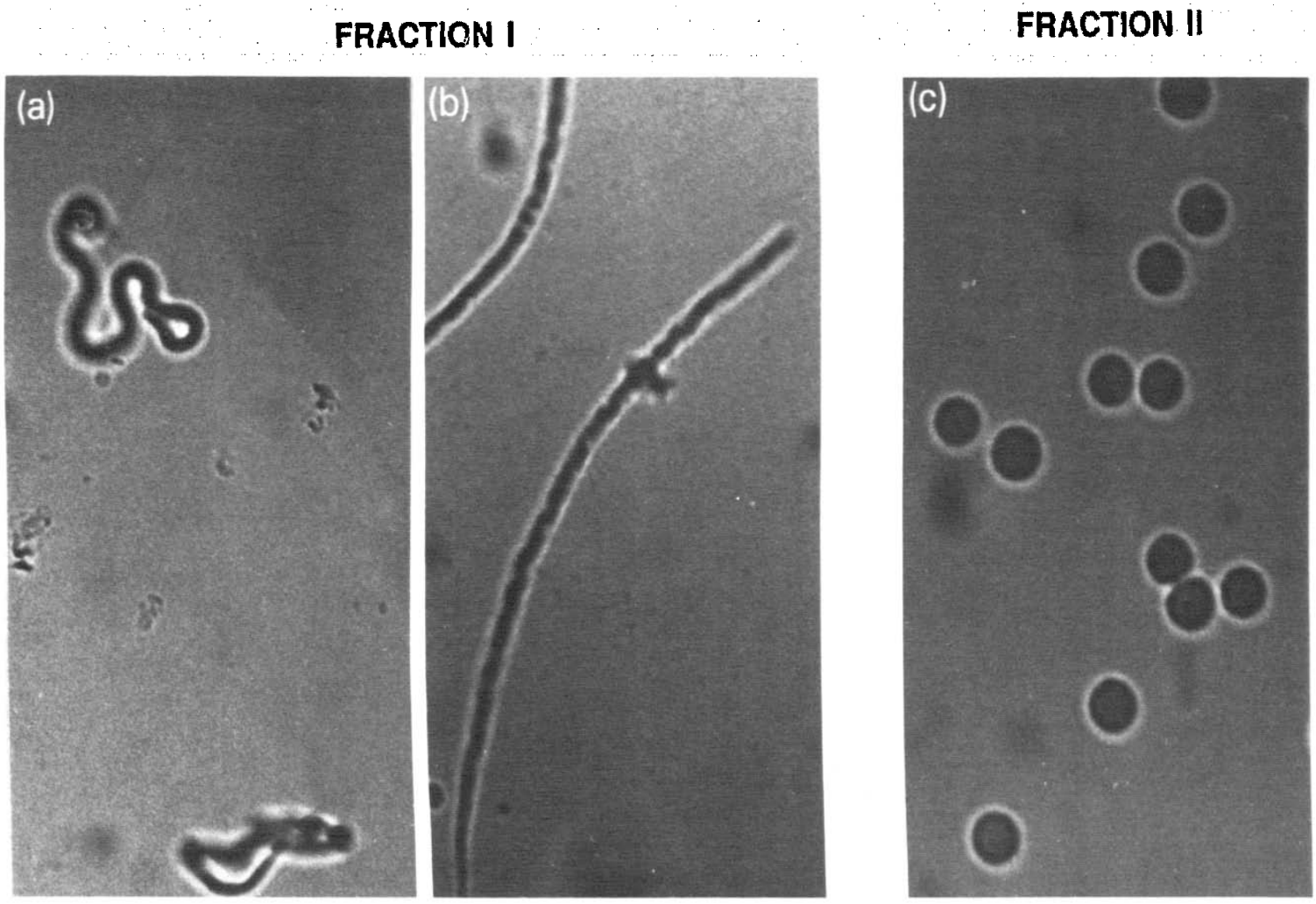

Fig. 2. Aspects of the species present in fractions I and II collected during the elution of rodent blood sample. Fractions I and II are shown in the elution profiles of Fig. 1. (A) and (B) mf observed with an optical microscope after collection of fraction I, in the case of live (A) and dead (B) mf. (C) Rodent RBC collected in fraction II in both cases. The injection protocol and elution conditions are identical to those in Fig. 1. (A and B) Drawn under optical microscopic observation, magnification ×100. (C) Microscopic observation, magnification ×400.

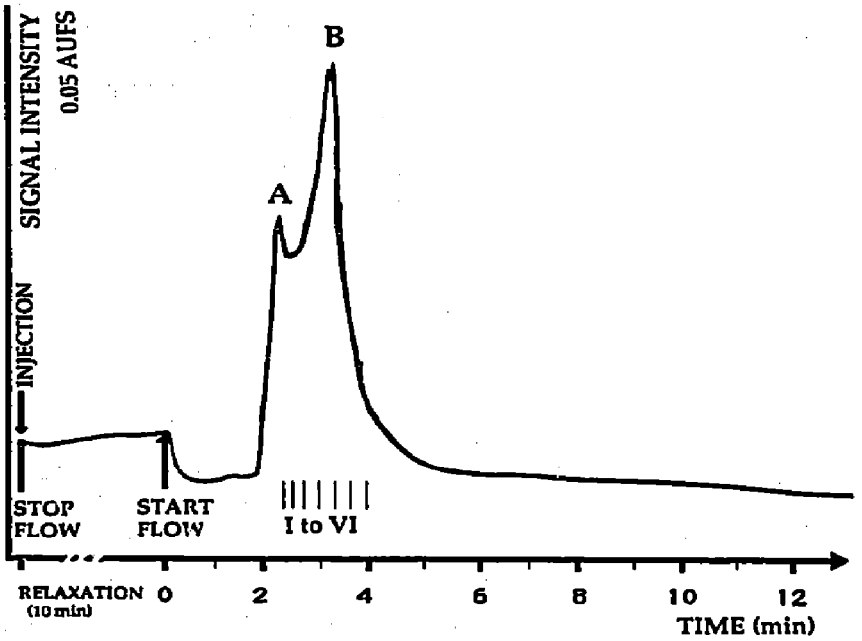

Fig. 3. Elution profile of the reinjection of microfilariae obtained after cumulative collections of fractions I of blood samples. A 40-μl aliquot of the pooled suspension was injected with a 10-min relaxation protocol (151 mf per 100 μl). Fraction I–VI were collected under time control and the mf counted. The elution conditions were identical to those in Fig. 1.

mf in a protein solution. After concentration, the suspension of mf was reinjected into the channel. In order to determine the elution position of mf, three elutions of that suspension were performed under the same experimental conditions. At each elution, a slicing of the two first peaks was performed and 200-μl fractions were taken, as indicated in Fig. 3. In each experiment the mf were counted and the average value of each fraction evaluated. The results are given in Table I. This series of experiments demonstrates the absence of mf in the first peak and the

TABLE I

MICROFILARIA CONCENTRATIONS IN THE COLLECTED FRACTIONS

The suspension collected in this experiment was obtained by pooling the mf fractions collected in the dead volume (fraction I) as presented in Fig. 1B and contained finally 151 mf per 100 μl. Three injections of 40 μl were performed and the table presents the average values obtained.

Fraction No.	Number of mf in the collected fraction	Volume of collected fraction (μl)	Concentration of mf in the collected fraction (mf/ml)
I	0	150	0
II	1 ± 1	150	7 ± 7
III	19 ± 2	200	95 ± 10
IV	35 ± 4	200	175 ± 20
V	2 ± 1	210	10 ± 5
VI	1 ± 0.4	210	5 ± 2

maximum mf concentration in the void volume of the channel. The elution position of the mf is then clearly defined, and the absence of mf in the first peak is also established.

Preparative isolation of live and dead mf and application of the detection sensitivity

As characterization of mf can be only achieved by optical observation with a microscope, suspensions of known concentrations of live and dead mf were prepared. Two series of experiments were performed, one with live mf and the other with dead mf. The concentration of the live mf suspension was 151 mf per 100 μl and the concentration of the dead mf was 180 mf per 100 μl. It should be pointed out here that live and dead mf have the same retention characteristics. Different volumes of these suspensions were reinjected into the GFFF channel to evaluate the recovery of the mf. The results are presented in Table II. The recovery, which can be up to 81%, is better for live mf than for dead mf, but there is a concentration effect, with lower recovery the lower the concentration of mf in the injected sample.

DISCUSSION

Because of the size difference, separation was not really difficult to achieve. In a 0.175-mm-thick channel the mf are not retained enough to have a retention factor

TABLE II

RECOVERY OF MICROFILARIAE

The suspension of mf injected in this experiment was obtained by pooling the mf fractions previously eluted at dead volume, and the elution conditions were identical to those in Fig. 1.

Injection volume of suspension (μl)	Theoretical number of injected mf	Number of mf collected (mean ± S.D., $n = 4$)
Dead microfilariae (suspension concentration 180 mf per 100 μl)		
50	90	62 ± 2
40	72	45 ± 6
30	54	33 ± 3
20	36	27 ± 3
10	18	2 ± 0.5
Average yield 67 ± 6%		
Live microfilariae (suspension concentration 151 mf per 100 μl)		
50	76	58 ± 7
40	60	52 ± 4
30	45	40 ± 4
20	30	22 ± 3
10	15	3 ± 1
Average yield 81 ± 9%		

≤ 1, but the use of a higher channel can increase the separation time. Even when eluted in the dead volume of the separation system, the mf are separated from the RBC. The very low number of nucleated cells injected (0.2%) and the simple carrier phase (not buffered) do not allow a significant survival of platelets and of nucleated blood cells because of their lower resistance in the simple isotonic carrier phase used here. They can be present in either the first or the second peak of the fractogram. Microscopic observation did not indicate a significant pollution of the mf fraction by these types of cells. The fraction collections and reinjections show the ruggedness and the reproducibility of GFFF for the elution of biological material. The reinjection process clearly demonstrates the retention conditions of mf. Surprisingly, in spite of their size, they are not eluted in the first peak of the channel.

Steric elution mode of microfilariae

As the mf are assumed to follow a steric elution mode because of their size of the order of microns, we can calculate the effective size of the mf in several different ways. A sphere of equal volume would have a diameter of 16.5 μm and, according to a "theoretical full steric mechanism", a retention factor of 0.28 would be expected. On the other hand, the experimentally found retention factor of 1 would suggest an equivalent sphere radius as large as 29 μm. The ratio of the radii of these two "equivalent spheres" is 3.5. In the case of live mf, as shown in Fig. 2, the coiled form of the mf corresponds approximately to a sphere with a radius of 26 μm, which should result in a retention factor of 0.87. In view of the relatively low precision of the evaluation of sphere sizes (relative error 10%) a steric interpretation of the mf elution in GFFF seems to be well justified. The fact that the experimental characteristic volume is higher than expected may be explained by the coiling movement of the live parasite, which can modify the "sphere volume", and lead to a higher apparent volume. Surprisingly, dead mf, which do not have this "spheric" conformation, are eluted in the same retention volume. The most likely interpretation of this result in the possible deformation of the mf in the flow profile, leading to a sickle-hook form, characterized by a radius of 29 μm.

Recovery of live and dead microfilariae

The average recovery of life mf was over 81%, and all the eluted parasites were alive at the outlet of the channel. This result is important, particularly in biology, where separation is the first step in further studies. The use of antiseptic carrier phases or the selective destruction of possible contaminants in the elution volume of the mf can increase the selectivity and the security of the elution process (human immunodeficiency virus contamination). The relatively low recovery in the case of dead parasites can be explained by some parasite-apparatus interactions. In any case, no probable degradation products could be found in the elutions. The most likely origin of this phenomenon is an irreversible adsorption of

the parasites on glass, either in the injection syringe or in the channel. Such an interaction would be favored by the fact that dead mf do not show jerking movements. A partial destruction of some parasites in the elution process is also possible.

Application to diagnosis of microfilarial diseases and to preparative separations

For the diagnosis of filarial disease, the observation of only one mf is sufficient. Using Table II with the limit value of 15 mf eluted, *e.g.* three mf observed under the microscope in a volume equivalent to a blood concentration of 2 mf per μl, is enough to assume the diagnosis. As the usual mf concentration in the blood can be 400–1000 mf per 20 μl of total blood sample [10], the use of 25 μl of blood is enough to diagnose mf infection by GFFF in most cases. The analysis time is less than 10 min, the method can be automated and a selective detection is possible by the use of a simple selective color labeling of the DNA of the mf nuclei with acridine orange dye coupled to a fluorimetric detector. With this short purification step, and the good recovery of the parasites after elution, the cumulative collection of the mf fraction of different samples can lead to preparative mf suspensions. The mf are obtained free of nucleated blood cells and RBC. The collected mf, once stained, are always morphologically identical to the ones observed in thin or thick blood smears. The detection limit after microscopic observation can be as low as five mf in a 25-μl blood sample.

CONCLUSIONS

In the development of the FFF technique, GFFF has been mostly considered as a basis for theoretical studies. Particularly well suited for the elution of micron-sized particle, as is asymmetric flow FFF, it has wide application in biology, especially in cellular biology. Using FFF, parasitology, particularly because of its continuing wide interest all over the world, is a large field to be explored. Parasites of micron size may be purified, and there is great interest in the possibility of using FFF techniques to elute selectively, at low cost, quantities of species in a preparative way. The possibility of obtaining pure fractions of parasites free of contaminating cells is important for all fundamental studies in parasitology. In pharmacology, immunology or molecular biology, for developing new drugs, studying new treatments or to developing new vaccines, GFFF may be a new alternative technique. Its low establishment cost, simplicity and rapidity could be helpful where a simple diagnostic technique is needed.

ACKNOWLEDGEMENTS

We wish to thank dr M. Czok of this laboratory for helpful discussions and corrections of this report. We also thank the teaching team of the Micromecanic Class of the Lycée de Villegenis-Massy, France for the construction of the GFFF apparatus used in this article.

REFERENCES

1 World Health Organization, *Tropical Diseases Research: a Global Partnership; 8th Program Report*, World Health Organization, Geneva, 1987.
2 M. B. Franks and N. R. Stoll, *J. Parasitol.*, 31 (1945) 158.
3 D. T. Dennis and B. H. Kean, *J. Parasitol.*, 57 (1971) 1146.
4 K. D. Caldwell, Z. Cheng, P. Hradecky and J. C. Giddings, *Cell Biophys.*, 6 (1984) 233.
5 J. C. Giddings, *Sep. Sci. Technol.*, 13 (1978) 241.
6 Ph. Gayral, G. Gueyouche, C. Bories, P. Loiseau, P. Demersman, G. Lamotte and R. Royer, *Drug Res.*, 39 (1989) 226.
7 J. C. Gantier, J. Maccario, P. N. Phat, C. Gueyouche and P. Gayral, *Ann. Parasitol. Hum. Comp.*, 62 (1987) 241.
8 C. C. Muscoplat, J. V. Schoster, C. A. Osborne and D. W. Johnson, *Am. J. Vet. Res.*, 38 (1977) 2095.
9 J. C. Giddings, M. N. Myers, K. D. Caldwell and S. R. Fisher, in D. Glick (Editor), *Methods of Biomedical Analysis*, Vol. 26, Wiley, New York, 1980, p. 79.
10 J. Chandenier, C. Pillier-Loriette, A. Datry, M. Rosenheim, M. Danis, H. Felix, J. P. Nozais and M. Gentilini, *Bull. Soc. Pathol. Expot.*, 80 (1987) 624.