Excretion of ecdysteroids by schistosomes as a marker of parasite infection

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Ecdysteroids produced by schistosomes are released in biological fluids of infected hosts. In the sera, the concentration of ecdysteroids correlates with the permissiveness of the host to schistosome infection and its detection is available in the absence of positive parasitological tests. In the urine, ecdysteroid concentration decreases markedly after chemotherapy. 20-Hydroxyecdysone and its epimer were identified in the urine of infected patients using mass spectrometry. These data demonstrate for the first time that ecdysteroids are released by organisms. Moreover, they are potent molecules of parasite infection and can be used for parasite diagnosis.

Schistosomiasis *Body fluids* *Ecdysterone* *Mass spectrum analysis* *Radioimmunoassay*

1. INTRODUCTION

The parasitic worms which cause schistosomiasis (bilharziasis) in man or rodents belong to the genus *Schistosoma*, a somewhat unusual group of digenic trematodes. This parasite has a complex life cycle involving an intermediate host in addition to the definitive host in which they reach sexual maturity. Schistosomes are the only trematode in which sexes are separate in adult worms. During the mature stage, the females may lay several hundred eggs per day which cause irreversible sequelae (granuloma, liver fibrosis). Another characteristic of schistosomes is their adoption of the blood as their adult habitat.

The presence of ecdysteroids, the insect moulting hormones, has been reported in the trematode *Schistosoma mansoni* where it is assumed that they control development and reproduction as they do in arthropods [1,2]. As well as the biological role of these ecdysteroids at the parasite level, it would be interesting to follow their fate in the infected host. This report shows that appreciable amounts of ecdysteroids are released into both sera and urine of infected hosts (rodents, monkeys and man), where they can be quantified by radioimmunoassay (RIA). Moreover, two of the excreted ecdysteroids have been characterised using high-performance liquid chromatography (HPLC), RIA, gas chromatography (GC) and electron impact mass spectrometry (MS). This has led to the identification of 20-hydroxyecdysone and 5-epi-20-hydroxyecdysone. It should be borne in mind that schistosomiasis (bilharziasis) is a major health problem in 73 tropical countries, where the number of infected people is estimated to be about 300 million. Our findings show that measurement of ecdysteroid concentrations in blood and urine can be used to diagnose parasitic diseases on a large scale, and to monitor the efficiency of chemotherapy.

2. MATERIALS AND METHODS

2.1. *Infection of rodents*

Hamsters (a) (permissive) and rats (b,c) (non-permissive) were infected by exposure to cercariae of *S. mansoni* as in [3]. They received 2000 cer-

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cariae, except for one group of rats which received only 500.

Two wild monkeys (*Patas patas*), aged 2 years and weighing 5 kg, were infected 10 times at 2-week intervals by exposure to 500 cercariae of *S. mansoni* as in [3]. The infection was checked by immunodiagnosis [4,5] and parasitological diagnosis [6]. The two animals differed from each other since monkey A had eggs in the stools whereas monkey B did not, even 535 days after the first infection.

2.2. *Ecdysteroid assay*

One-ml samples of serum were taken and added to 3 ml methanol. The mixture was heated at 60°C for 15 min and centrifuged (800 × *g*, 10 min). The supernatant was dried under a flow of nitrogen. The residue was dissolved in 0.1 M citrate buffer (pH 6.1) and then assayed according to the RIA method in [7].

Urine samples: the study was conducted on 60 children aged 12–14 years from Niamey (Nigeria), infected with *S. haematobium*. Urine samples were taken around 10 a.m. at school. On the spot, 1 ml urine was added to 3 ml methanol and the mixture sent to our laboratory. Samples were then treated as described above.

2.3. *Chemotherapy*

The children received a single dose of antihelminthic drugs. The first group (n = 20) was treated with Oltipraz alone (30 mg/kg), the second (n = 20) with Oltipraz plus vitamins (30 mg/kg) and the last group (n = 20) received Praziquantel (30 mg/kg).

2.4. *Mass spectrum analysis*

Urine from 20 people infected with *S. haematobium* was collected (600 ml). Urine samples were added to 1200 ml butanol. After centrifugation at 1000 × *g* for 3 min, the butanol phase was dried under reduced pressure at 40°C. The residue was then dissolved in 95% ethanol before being centrifuged (1000 × *g*, 3 min). The supernatant was dried under nitrogen and suspended in methanol prior to being analysed by thin-layer chromatography (silica gel plates 20 × 20, F254, Merck) with chloroform–methanol as solvent (80:20, v/v). Bands of 1 cm width were scraped off and extracted with 95% ethanol. Immunoreactive samples were then analysed by HPLC as in [2]. GC was performed on immunoreactive HPLC fractions after derivatization. For this purpose, 100 μl trimethylsilylimidazole (TSIM) were added. This solution was heated overnight at 80°C under argon, and separation of derivatized ecdysteroids from the reagent was performed by HPLC on a silica column with ethanol–hexane as solvent. After evaporation of the solvent under argon, acetone was added to the dried extract and 1–2 μl were injected into a GC apparatus (Varian 1200, capillary glass column SE/30) at 290°C. An LKB 9000 S electron impact mass spectrometer was coupled to the gas chromatograph. This spectrometer was equipped with an Incoss computer system which allows the recording of one mass spectrum per s during the whole process of chromatography.

3. RESULTS

Fig.1 shows the results of ecdysteroid RIA measurement in the blood of rodents before and after infection with *S. mansoni*. It is apparent that no immunoreactivity is recorded before infection and during the first 5 days after infection.

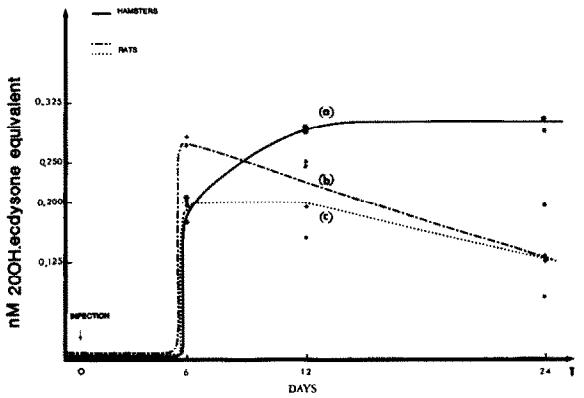


Fig.1. Changes in ecdysteroid concentration in the blood of rodents before and after infection. Hamsters (permissive) and rats (non-permissive) were infected with cercariae of *S. mansoni*. Blood samples were analysed by RIA on different days of infection. (a) Hamsters infected with 2000 cercariae, (b) rats infected with 2000 cercariae, (c) rats infected with 500 cercariae. Each experimental point is the mean of 4 individual values; bars, SE.

However, on day 6, appreciable amounts of ecdysteroids can be detected in the blood of the rodents. The changes in hormonal level differ between permissive (hamsters) and non-permissive hosts (rats). In the first case, the ecdysteroid concentration reaches 0.3 nM on day 12 post-infection and this value is unchanged on day 24 (fig.1a). In contrast, in non-permissive hosts, the ecdysteroid level reaches 0.3 nM as early as day 6 and, more importantly, decreases thereafter, reaching 0.22 nM on day 12 and 0.13 nM on day 24 (fig.1b). Fig.1 also shows that the rise in ecdysteroid concentration which occurs on day 6 is significantly lower in rats which were challenged with a dose of 500 cercariae compared to rats challenged with 2000 cercariae (fig.1c). On the following days, the two groups of rats did not differ significantly.

Ecdysteroids are also present in the blood of infected primates. Fig.2 shows that, in *P. patas* monkeys, they reach 4 nM in the serum on day 218 post-infection and more than 8 nM on day 499 and day 535. In contrast, ecdysteroid levels in an uninfected monkey were below 0.3 nM in the serum (fig.2c). It should be noted that *S. mansoni* eggs were found in the stools of monkey A during the duration of the experiments (10–180 eggs/g). Monkey B lacked eggs in the stools on day 499 and thereafter. Despite this difference in the form of the chronic model, ecdysteroid levels were very similar in both animals.

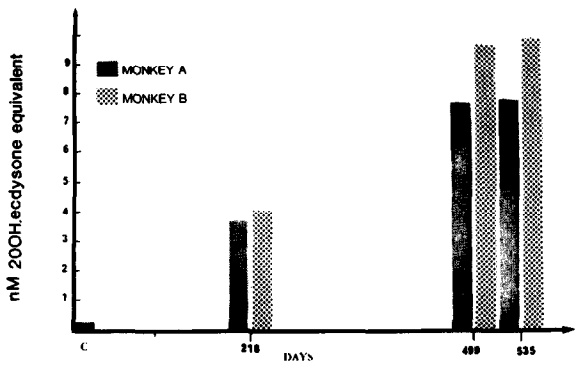


Fig.2. Ecdysteroid titer in sera of monkeys infected with *S. mansoni*. Monkey A, chronic model of infection with eggs in the stools during infection. Monkey B, chronic model of infection without eggs following day 218. (c) Control performed with blood of uninfected monkeys treated under the same conditions.

Ecdysteroids were assayed in urine of children infected with *S. haematobium*. Patients had very high concentrations of these hormones; the mean level was 30 nM and the highest value found in 3 children was 180 nM (fig.3). It is noted that the hormonal concentration decreased after antihelminthic chemotherapy. Four days after drug administration, the ecdysteroid titer fell to one-third, and then slowly decreased, reaching around 6 nM some 100 days after treatment. Chemical characterization of ecdysteroids found in urine of infected patients was achieved by GC and MS after trimethylsilylation (fig.4). Total ion recording and fragmentograph ion recording are shown in fig.4A. The total ion analysis (fig.4A,a) indicates that several molecules are present in urine samples. The fragmentograph recording of the main ion of 20-hydroxyecdysone at *m/e* 561 (fig.4A,b) [8] allows the detection of two major molecular forms. The first peak (vertical arrow 1) has the same retention time as standard hexatrimethylsilyl 20-hydroxyecdysone and the second (arrow 2) has the same retention time as standard hexamethylsilyl 5-epi-20-hydroxyecdysone (reference compound prepared by Dr Hetru [9]) run under the same conditions. The mass spectrum of these

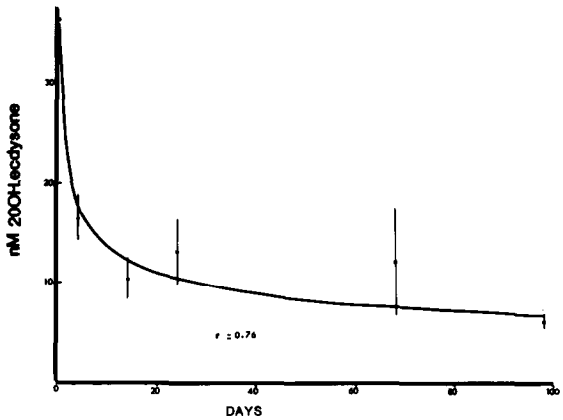
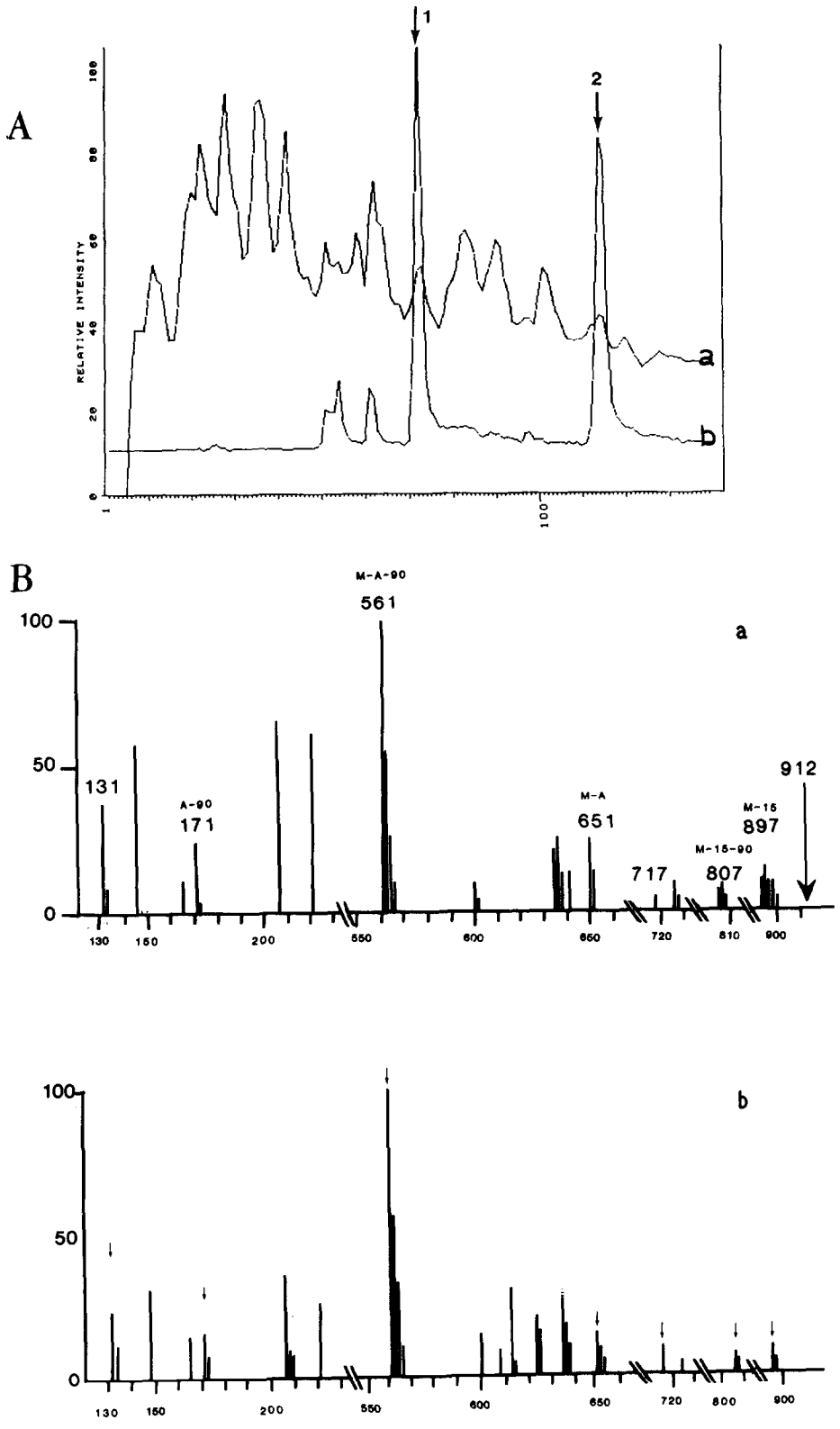


Fig.3. Changes in ecdysteroid titer in urine from patients infected with *S. haematobium* before and after chemotherapy. On day 0 the children received a single dose of antihelminthic drugs: Oltipraz alone or Oltipraz plus vitamins or Praziquantel. The effect of these treatments on the ecdysteroid titer was very similar, therefore the means of 60 individual values are shown (each individual value is the mean of triplicates); bars, SE.


A
1
2
RELATIVE INTENSITY
a
b
B
M-A-90
561
a
131
A-90
171
M-A
651
717
M-15
897
M-15-90
807
912
b

molecules is given in fig.4B. The spectrum of reference trimethylsilylated 20-hydroxyecdysone (fig.4B,a) has the main ion at *m/e* 561 which corresponds to the loss of the side chain of the ecdysteroid after cleavage between C-20 and C-22 and the loss of a silanol in the case of fully silylated derivatives. This ion is highly characteristic of the hexatrimethylsilylated 20-hydroxyecdysone. The fragments at *m/e* 131 and 171 correspond to fragmentation of the 20-hydroxyecdysone side chain [10]. Moreover, the fragment at *m/e* 897 (molecular ion minus one methyl) is also present. In conclusion, peaks 1 and 2 yield the same mass spectra which are similar to the mass spectrum of 20-hydroxyecdysone obtained under the same conditions. The difference in retention time is explained by the fact that peak 2 corresponds to the 5-α epimer of 20-hydroxyecdysone.

4. DISCUSSION

This study demonstrates that ecdysteroid hormones present in schistosomes are released into the sera and urine of infected hosts. This is true for experimental rodent hosts as well as for primates and man. These ecdysteroids are present in sera of infected rodents as early as 6 days post-infection. The time course of the ecdysteroid titer shows a good correlation with the susceptibility or the innate resistance of the host to schistosome infection. The ecdysteroid level is maintained in hamsters whereas it decreases in rats. This important observation suggests that the concentration of ecdysteroids is an early biological parameter of parasite infection. The presence of ecdysteroids in urine and sera of infected vertebrates seems to be pertinent in the follow up of the disease. The time course of the ecdysteroid concentration might also be correlated with the viability of the parasites. To support this concept, the appreciation of ecdysteroid titer from urine of infected humans allows the detection of a dramatic decrease after antischistosome chemotherapy. In addition, gas chromatography and electron impact mass spectroscopy studies on the same human material demonstrate that two main ecdysteroid molecules are present: 20-hydroxyecdysone and 5-epi-20-hydroxyecdysone. Furthermore, other minor molecules which are immunoreactive have been detected, but their structures have not yet been identified. Moreover, these ecdysteroids can be detected even in the absence of parasitological markers, i.e., eggs in the stools, as stated above for *P. patas* monkeys.

From a clinical point of view, the presence of ecdysteroids in the sera and urine of infected humans is of practical interest because they can be used as a biological marker of parasite infection and thus palliate 3 drawbacks of parasitological diagnosis which are:

(i) No detection before the late egg-laying stage of the parasites;
(ii) Decrease or absence of egg laying in chronic infection;
(iii) Relative unreliability of the parasitological tests.

In addition, except for the classical parasitological tests, no other biological test allows an accurate determination of the efficiency of chemotherapy.

Together with new insights into host-parasite relationships (i.e., immunological system or endocrine system), this finding opens novel approaches for early detection of parasitic diseases and for monitoring the efficiency of therapy.

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Fig.4. Mass spectrum of hexatrimethylsilylated natural compounds present in urine of patients infected with *S. mansoni*. (A) Total ion gas chromatogram: (a) total ion recording, (b) recording of ion fragment at *m/e* 561. (B) Mass spectra: (a) mass spectrum of hexatrimethylsilylated reference 20-hydroxyecdysone (reference from SIMES, Milan), (b) mass spectrum of endogenous ecdysteroid yielding peak 1 in the fragmentogram of part A (the mass spectrum of the endogenous ecdysteroid yielding peak 2 in the fragmentogram is not shown as it is identical to that of peak 1).

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