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Disposition of Nifurtimox and Metabolite Activity Against *Trypanosoma cruzi* using Rat Isolated Perfused Liver

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Abstract—Nifurtimox disposition was investigated using the rat isolated perfused-liver method after administration of 25 $\mu g\ mL^{-1}$ nifurtimox, and its disappearance was monitored by analysing the perfusate sample at various times. Biliary excretion was also measured. The drug concentration profile underwent a biexponential decline over the 2-h study period, with a terminal half-life of $62{\cdot}76 \pm 17{\cdot}56$ min. Nifurtimox is a high clearance compound ($15{\cdot}23 \pm 5{\cdot}53$ mL min^{-1}). The extraction ratio was $0{\cdot}621 \pm 0{\cdot}159$. Biliary excretion accounted for 0·05% of the dose, the remainder consisting of highly polar metabolites. By 2 h, a minimal fraction of unchanged nifurtimox was recovered from the perfusate. Nifurtimox activity against *Trypanosoma cruzi* (clone CA-1) during the perfusion was also determined. Epimastigotes isolated from continuous culture were exposed to the samples of perfusate at different perfusion times in a microtitre plate. After an incubation time of 72 h at 27°C, the parasite number in each well was counted under a microscope. From 0 to 75 min after the perfusion, the anti-trypanosomal activity decreased, but an increase in activity was observed at the later times. These findings show that active metabolites are formed during the perfusion.

Nifurtimox is a drug widely used in Latin-American countries for the treatment of Chagas' disease. In a previous study (González-Martin et al 1993), a rat perfused-liver method was used to show that this drug exhibits extensive hepatic metabolism. In that study, the drug was rapidly cleared from the perfusate fluid with an elimination half-life of approximately 50–60 min, and a hepatic clearance of 3·5 mL min^{-1}. These results could partially explain the low plasma concentration found in two different pharmacokinetic studies carried out on healthy volunteers (Paulos et al 1989) and in chronic renally impaired patients in a programme of haemodialysis (González-Martin et al 1992) after oral administration of nifurtimox at a dose of 15 mg kg^{-1}. In those reports it was suggested that the lower serum level of nifurtimox could be due to an increased hepatic first-pass effect and a rapid transformation of the drug in the liver. This rapid metabolism could give rise to drug metabolites with trypanocide activity, thus, potentiating the action of the drug and, additionally, contributing to the toxicity that nifurtimox presents in man.

In a previous study (González-Martin et al 1993), we used a recirculation method without cannulating the bile duct. Accordingly, nifurtimox concentrations and pharmacokinetic parameters may not have reflected the biliary elimination of this drug. There is information that other nitrofuran compounds such as nitrofurazone (Sorrentino et al 1987) appear to be conjugated and eliminated extensively in bile.

The aim of the present study was to determine the pharmacokinetic profile of nifurtimox using a rat isolated-liver technique with bile cannulation, and to determine trypanocide activity at different perfusion times so as to obtain information on the possible contribution of the metabolites formed during perfusion to the drug trypanocide activity.

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Materials and Methods

Chemicals

Nifurtimox was a gift from Bayer Laboratories (Buenos Aires, Argentina) and carbamazepine was a gift from Recalcine Laboratories (Santiago, Chile). All solvents were HPLC grade and other reagents and chemicals were purchased from Merck Química Chilena, Santiago, Chile.

Animals

Male Sprague-Dawley rats, 140–200 g (Instituto de Salud Pública, Santiago, Chile), were housed in well ventilated cages and kept at a room temperature of approximately 24°C. They were allowed free access to pelleted food and tap water until 24 h before surgery. Animals were cared for in accordance with the principles of The Guide for the Care and Use of Laboratory Animals (Governmental Activities Relating to the Use of Animals in Research 1985).

Isolated perfused livers

Rats were anaesthetized with urethane (1·5 g kg^{-1}, i.p.). The abdominal cavity was opened and the bile duct cannulated with Teflon tubing (0·6 mm o.d.). Next, the portal vein and the thoracic inferior vena cava were cannulated. The liver was cut free and placed in a humidified and thermoregulated cabinet. The perfusate was pumped (Masterflex, Cole Palmer, Chicago, IL, USA) through a filter, to a membrane lung (Silastic medical grade tubing, Dow Corning, Midland, MI, USA) where it was oxygenated with 95% O_2/5% CO_2, over a 37°C thermoregulated bath, then to a bubble trap before reaching the liver. In the recirculating mode, the perfusate exited through the inferior vena cava cannula and

dropped back into the glass reservoir before returning to the pump.

The perfusate consisted of 500 mL of a modified Krebs–Henseleit bicarbonate buffer containing 1 mg mL^{-1} glucose. The perfusate was divided into two flasks each containing 250 mL. Nifurtimox dissolved in 2 mL dimethyl sulphoxide was added to one of these flasks to a final concentration of 25 $\mu g\ mL^{-1}$. The flow rate was adjusted to about 3 mL min^{-1} g^{-1} assuming a liver weight of 3·5% total body weight. Constant perfusate pH of 7·4 was maintained throughout the experiment.

The liver was perfused for 120 min with nifurtimox in the recirculating mode after an equilibrium period of 30 min in the single-pass mode with blank perfusate. Four millilitres of efflux perfusate was collected at 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. For each time point, 2 mL perfusate was used to measure the activity against *Trypanosoma cruzi*, and another 2 mL for nifurtimox assay. The samples were frozen until the assay. It was established that nifurtimox did not bind to the experimental apparatus (injector, tubing, cannulas or collection apparatus) used in the perfusion studies. For the study five livers were perfused.

Nifurtimox assay

Two millilitres perfusate was mixed with 100 μL internal standard solution (carbamazepine, 250 $\mu g\ mL^{-1}$) dissolved in dimethyl sulphoxide and extracted by passing through a SepPack C_{18} cartridge (Waters Associates, Inc.) fitted to a Luer Lock glass syringe. The SepPack C_{18} cartridge was prepared by flushing with 2 mL methanol. The perfusate samples were passed through the cartridge and washed with 5 mL distilled water. Unchanged nifurtimox was eluted with 2 mL methanol. The methanol fractions were evaporated to dryness under nitrogen. The residue was dissolved in 500 μL mobile phase (methanol-phosphate buffer pH 7·0, 50:50). Recovery was 97·0%. Twenty microlitres was injected on to a Shimadzu LC-9A model pump. Chromatographic separation was achieved using a Merck LiCrospher 100 RP-18 (5 μm) in LiChroCART 125-4 (4·6 × 12·5 mm). Retention times for nifurtimox and internal standard were 2·8 and 6·2 min, respectively. A Shimadzu UV-spectrophotometer, set at 270 nm for the detection of the nifurtimox and carbamazepine, was used. The assay was specific for nifurtimox. Coefficients of variation were (within day) 5·0% at 15 $\mu g\ mL^{-1}$.

Pharmacokinetic calculations

The perfusate concentration-time data for nifurtimox were fitted using a two-compartment model and are described by the equation:

$$c_t = Ae^{-\alpha t} + B^{-\beta t}$$

in which c_t is the nifurtimox concentration of perfusate at time t, and α and β are functions of all three rate constants: k_{12}, k_{21}, and k_{el}. A and B are the zero-time intercepts of the extrapolated lines of the faster and slower components. These pharmacokinetic parameters were calculated from the equations of Wagner (1975) and Gibaldi & Perrier (1975).

The extraction rate was calculated as clearance divided by flow rate.

Measurement of metabolites in the perfusate

The metabolites obtained during perfusion were not identified. Therefore, a relationship between the areas of nifurtimox and metabolites was calculated from the HPLC record, assuming equivalent UV absorbance.

Parasite

Trypanosoma cruzi was isolated from a chronic Chagas' disease patient from northern Chile and was originally obtained from Dr Aldo Solari from the Biochemistry Department, Faculty of Medicine, University of Chile. Multiple clones were prepared, and after several passages in rats, one clone (CA-1) was finally adapted for axenic cultivation. Epimastigote forms were used to evaluate the activity against *T. cruzi*.

Culture medium

Minimal essential medium (MEM; Gibco-BRL 072-110) with Hanks' salts (Gibco-BRL 041-01575) supplemented with 10% calf bovine serum was used to maintain the epimastigote forms. Cultures were maintained in T-25 culture flasks (Sterilin) at 28°C and then subpassaged at 10^4 cells mL^{-1} every third day. For sensitivity determinations, trypanosomes in the logarithmic growth phase were taken from these stock cultures.

Test procedure

Samples obtained during perfusion were filtered through a 0·22 μm sterile membrane and 75 μL of each was placed in each well of row A and row D of a microtitre plate (Nunc, InterMed, Denmark).

All remaining wells of the plate received 50 μL medium. For threefold serial dilutions, 25 μL from each well of row A and D was transferred to the corresponding well of row B and E and this was continued up to the wells of row C and F for which 25 μL was discarded. Columns A11 and A12 served as controls.

Fifty microlitres of medium containing 10^6 cells mL^{-1} was added to all wells to give a final concentration 5×10^5 cells mL^{-1}. Medium alone (50 μL) was added to the wells of the remaining rows, which served as additional cell-free controls. Culture dishes were placed in an incubator at 28°C in a 5% CO_2 atmosphere for 72 h. To prevent evaporation, dishes were kept in a humid chamber in the incubator.

To determine the activity of the unchanged nifurtimox, 75 μL nifurtimox standard solution (50 $\mu g\ mL^{-1}$) was added to the wells of rows A1 to A3 in another microtitre. Then, the same dilutions were performed.

Parasite counting was performed in a Neubauer chamber (Boeco, Germany) under a microscope (Cambridge Instruments, Galen III).

Calculation of activity

T. cruzi growth inhibition at each perfusion time was calculated using the equation:

$$\%\ \text{activity} = \frac{N_b - N_w}{N_b}$$

where N_b is the number of parasites in the blank and N_w is the number of parasites in each well.

Table 1. Biexponential parameters in rat perfused livers after 25 μg mL^{-1} nifurtimox.

Perfusion No.	A (μg mL^{-1})	α (min^{-1})	B (μg mL^{-1})	β (min^{-1})
1	23·73	0·0758	1·59	0·0990
2	16·96	0·0412	4·40	0·0123
3	20·09	0·0625	1·74	0·0218
4	28·26	0·0689	1·23	0·0095
5	20·87	0·1251	1·62	0·0084
Mean	21·98	0·0747	2·12	0·0124
±s.d.	4·21	0·0129	1·26	0·0049

Table 2. Pharmacokinetic parameters of nifurtimox in rat perfused liver.

Parameters	Mean ± s.d.
Volume of distribution (mL)	296·2 ± 73·41
Rate constants (min^{-1})	
k_{10} (elimination)	0·0505 ± 0·0114
k_{12} (distribution)	0·0193 ± 0·0051
k_{21} (distribution)	0·0173 ± 0·0050
Area under curve (μg min mL^{-1})	509·13 ± 138·41
Clearance (mL min^{-1})	15·23 ± 5·53
β Half-life (min)	62·76 ± 17·56
Extraction ratio (E)	0·621 ± 0·159

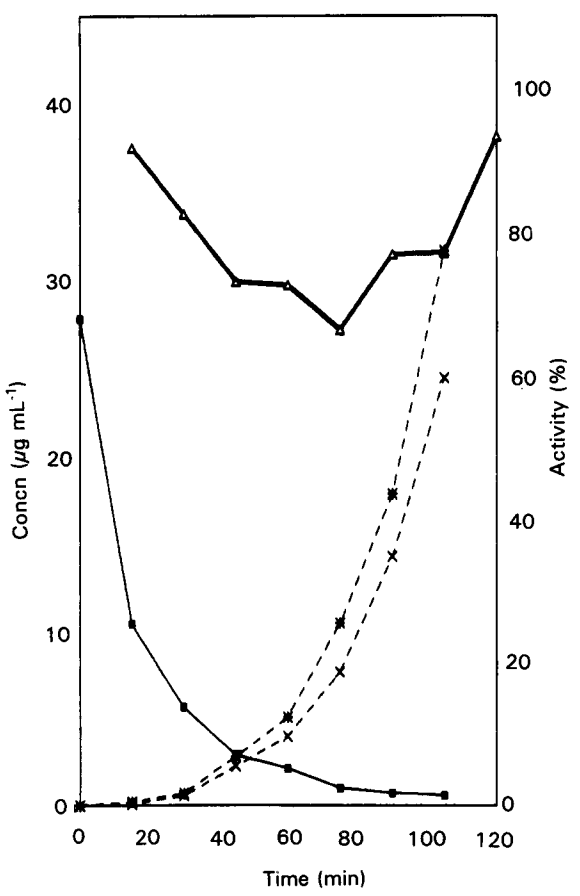

FIG. 1. Average of nifurtimox concentrations (■), increase of area ratios of two metabolites (*, ×), and percentage of activity against *Trypanosoma cruzi* (△) in perfusates.

Table 3. Activity against *Trypanosoma cruzi* (%) in rat perfused livers.

Perfusion No.	Time (min)								
	0	15	30	45	60	75	90	105	120
1	80	80	57	72	71	79	81	93	96
2	100	100	88	76	85	58	53	41	81
3	84	78	72	85	81	73	86	100	100
4	100	100	100	73	62	38	80	67	100
5	100	99	95	45	63	83	84	83	89
Mean	93	92	82	70	72	66	77	77	93
±s.d.	9	10	16	13	9	16	12	21	7

Results

Nifurtimox pharmacokinetics

The biexponential parameters A, α, B, and β, were computer-fitted to the disposition curve for five perfused livers, using a nonlinear least-square method program (PCNONLIN) (Metzler & Weiner 1985). These results (Table 1) were used to calculate the numerical values for the parameters of the biexponential decay (Table 2).

The value of β half-life was 62·76 ± 17·56 min, similar to

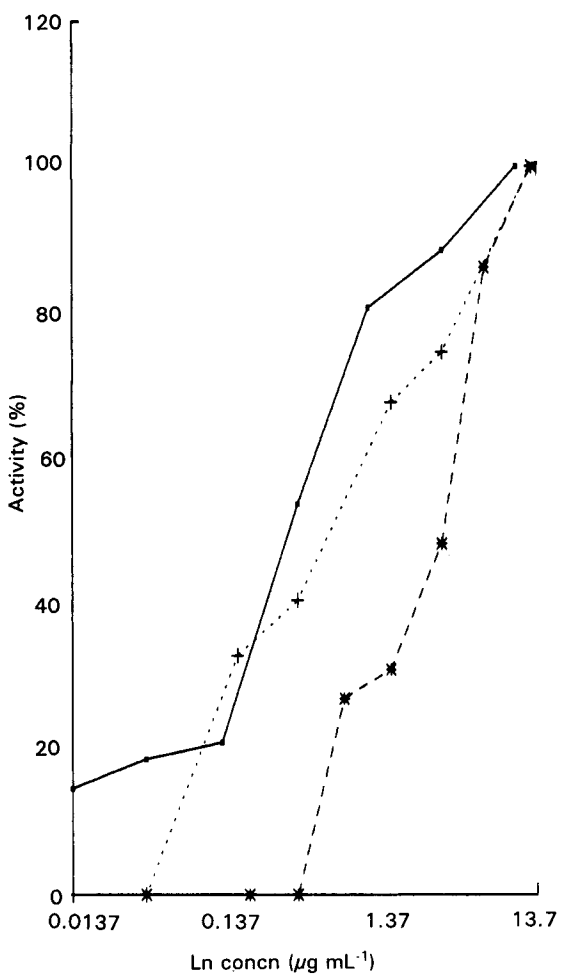

FIG. 2. Percentage of activity against *Trypanosoma cruzi* vs logarithm of nifurtimox concentrations after three different trials.

the value obtained in a previous study (González-Martin et al 1993). Hepatic clearance was $15{\cdot}23 \pm 5{\cdot}53$ mL min^{-1}, comparable with the perfusate rate (20 mL min^{-1}), and the extraction rate was 0·621. These values demonstrate that nifurtimox has an extensive liver first-pass effect and a high percentage of nifurtimox is converted to metabolites. The HPLC traces showed two peaks with retention times of 0·70 and 0·94 min that were not present in the first minutes of perfusion. Increases in height and area of these peaks were observed with increase in perfusion time and decrease in nifurtimox concentrations. The ratios of these possible metabolite areas in relation to nifurtimox area are shown in Fig. 1.

A small amount of unchanged nifurtimox was found in the bile (< 10 μg mL^{-1}). Numerous products related to nifurtimox were present in bile as observed on the HPLC traces after the injection of a bile sample. These unidentified products could be metabolites or conjugated products obtained during the liver perfusion.

Trypanocidal activity

Table 3 shows activity against *T. cruzi* of perfusate samples. From 0 to 75 min after the perfusion, the activity decreased. However, an increase in activity occurred at the end of the perfusion. This finding was not correlated with nifurtimox concentrations, since the activity of the drug decreases as nifurtimox concentrations decay (Fig. 2).

Fig. 1 shows average nifurtimox concentrations (μg mL^{-1}), the increase of the area ratio of the metabolites, and the percentage of activity against *T. cruzi* at different perfusion times. The activity did not reach zero at any time during the perfusion, thus showing that the metabolites formed contribute to the activity of the drug. A blank perfusion verified that non-drug related hepatic products with trypanocidal activity did not arise during the perfusion.

Discussion

In the present study, important changes in pharmacokinetic parameters of nifurtimox were found in relation to a previous study carried out at our laboratory using the same technique of rat isolated liver perfusion (González-Martin et al 1993), but without collection of bile.

The increase in extraction ratio in this study supports the hypothesis that the drug undergoes significant metabolism and it is subjected to fast elimination in the liver. These results agree with studies performed by Duhm et al (1972) and Medenwald et al (1972), where it was shown that nifurtimox is extensively metabolized in animals; only 0·5% of a single oral dose of nifurtimox was recovered as unchanged drug in the urine of rats and dogs.

Nitrofurazone, another nitrofuran compound which has a similar chemical structure to that of nifurtimox, is significantly eliminated in the bile. Previous reports (Sorrentino et al 1987) indicate that nitrofurazone is excreted in bile largely as a glutathione or cysteine-glycine conjugate.

The high liver first-pass effect of nifurtimox, reflected by the high extraction ratio in this study, could explain the low plasma level observed in healthy volunteers (Paulos et al 1989), and in patients with chronic renal failure (González-Martin et al 1992) after oral administration of 15 mg kg^{-1}, reported in two previous studies carried out in our laboratory.

*Anti-*T. cruzi *activity*

The most significant finding of this study is the variation in the anti-*T. cruzi* activity of nifurtimox shown during perfusion. It is apparent that initial decay of nifurtimox concentrations leads to decreased activity against *T. cruzi*. However, this decrease is not continued as perfusion time increases. Thus, after 75 min of perfusion the activity begins to increase, reaching the activity levels at the onset of perfusion by 120 min.

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