*Journal of Radioanalytical and Nuclear Chemistry, Vol. 269, No.3 (2006) 579–583*

# Biodistribution of gyroxin using $^{125}I$ as radiotracer

**J. A. Alves da Silva,[1]* E. Muramoto,[2] M. T. C. P. Ribela,[1] J. R. Rogero,[1] M. A. P. Camillo[1]**

[1] *Molecular Biology Center, IPEN/CNEN-SP, P.O.Box 11049, 05422-970, São Paulo, Brazil*
[2] *Radiopharmacy Center, IPEN/CNEN-SP, P.O.Box 11049, 05422-970, São Paulo, Brazil*

(Received April 6, 2006)

The use of radiotracers in the research of animal venom has been scarce, although it allows an excellent approach to follow the process of bioavailability, biodistribution and kinetics of toxins. The purpose of this study was to assess gyroxin action mechanism, transport, compartments and action sites. This toxin is a thrombin-like and causes the barrel rotation syndrome. The gyroxin was labeled with $^{125}I$ and used as a tracer for the in vivo assay in mice. Blood samples and organs were collected at different time intervals, weighed and analyzed in a gamma-counter. The data was related with tissues distribution of protease activated receptor (PAR). Biodistribution assay allowed dividing the organs into three groups. The first one with the organs that followed the blood kinetics, the second with the organs related to metabolisms and elimination, and the third with the organs in which the gyroxin concentration increased during the observation period.

## Introduction

The distribution, metabolism and excretion (DME) of a strange compound in a living organism depends on physicals, chemicals and physiological factors (pharmacokinetics processes). These factors remove and dilute the compound from its place of entrance in the biological system, carry it to different tissues, allow its diffusion or promote its transportation actively through cells membranes and finally determine its accumulation, disposition and excretion. The biodistribution rate in the organ's tissue is determined by the blood flow perfusion and by the ability of the compound molecule to pass through the capillary wall and penetrate into the cells.[1]

Gyroxin is a serine protease present in the venom of *Crotalus durissus terrificus*, a Brazilian rattlesnake. It was first described as a neurotoxin causing motor disturbances called barrel rotation.[2,3] The intravenous (IV) injection of gyroxin into mice produces temporary episodes characterized by opisthotonos and rotations around the long axis of the body: that is the reason why it is called gyroxin. Gyroxin is also known for its thrombin-like activity on human fibrinogen, cleaving the alpha-chain.[4] The fibrin monomers polymerizes into an abnormal fibrin clot and it is highly susceptible to the action of fibrinolytic agents.[5,6] The total or partial incoagulability observed in the blood of patients bitten by rattlesnakes derives from the fibrinogen consumption.[7]

There is a special interest in gyroxin action on fibrinogen because of its possible use in thrombotic disease therapy and as an anticoagulant factor. Some thrombin-like enzymes isolated from the venom of other snakes are being used as defibrinogenating agents for a number of clinical conditions including deep vein thromboses, myocardial infarction, pulmonary embolus, acute ischemic stroke and peripheral vascular disease.[5]

As told before, gyroxin is a multifunctional protein but its action mechanism is not well-known, especially regarding neurotoxicity. This work aims at investigating gyroxin action mechanism, transport, compartments and action sites using $^{125}I$ as radiotracer.

The animals' toxins could be radiolabeled for biodistribution assays, indirectly or directly:

Indirectly, by labeling the toxin in an already radiolabeled molecule (e.g., an antibody). This incorporated radiotracer will show the toxin metabolism in a living organism during the whole experimental period.

Directly, by incorporating the radionuclide to the protein molecule, cofactors, coenzymes or metal, when one of these constituents is present. The directly way was used in this study (gyroxin was radiolabeled with $^{125}I$).

Radiolabeling with $^{125}I$ has been indicated for several applications due to its easy and simple radioiodination system, labeled product stability and detection facility.[8]

## Experimental

### *Gyroxin radioiodination*

Gyroxin was obtained from *Crotalus durissus terrificus'* crude venom by affinity and gel filtration chromatography.[9] It was radioiodinated using mild conditions at low temperature with chloramine-T method.[10] The reaction mixture were fractionated in a Sephadex G100 column (3×50 $cm^2$) with 50mM sodium phosphate buffer pH 7.4 containing 0.1% of bovine serum albumin as eluent. Fractions of 2.0 ml/tube were collected and the flow rate was 12 ml/h. Aliquots with 20 µl from each tube were analyzed in a gamma-counter (Oakfield Instruments, United Kingdown).

* E-mail: josilva@ipen.br

*0236–5731/USD 20.00*

*Akadémiai Kiadó, Budapest*
*Springer, Dordrecht*

The [$^{125}$I]gyroxin obtained was analyzed in non-denaturating electrophoresis system.[11] The plate gel (15%) was cut first in vertical slices to separate the bands and then in 5 mm horizontal slices. Each slice was analyzed in a gamma-counter.

*In vivo assay*

This study was analyzed and approved by Brazilian Ethics and Animal Experimentation Committee – Colégio Brasileiro de Experimentação Animal (COBEA).

Thirty B10.PL isogenic male adult mice weighing about 20 g were used to analyze the [$^{125}$I]gyroxin biodistribution. They were divided into 10 groups with 3 animals each. The [$^{125}$I]gyroxin ($8.3 \cdot 10^5$ Bq/100 µl) was injected by intravenous via in the tail vein. All animals were maintained in cages covered with pinewood sawdust and fed with commercial food and water ad libitum. After different time intervals (3, 5, 15, 30, 60, 180, 360, 600, 1500, 2880 minutes), the animals were anesthetized to collect blood sample with Pasteur pipette, pre-treated with heparin, and sacrificed. The organs (brain, heart, lung, liver, kidney, spleen, stomach, thyroid, intestine, skeletal muscle and tail) were removed, weighed and analyzed in a gamma-counter. The radioactivity found in the tail was subtracted from the total injected dose. The final results were analyzed by Excel software and showed as percentage of total dose to organ body mass (%Dose/g). The results were then presented with two graphical software. The first compared the percentage of total dose incorporated by body mass (%Dose/g) in different organs in each time intervals studied. The second software, (GRAPH PADPRISMA) expressed the percentage of total dose to organ body mass (%Dose/g) as a function of time, in a XY-graphic model.

## Results and discussion

The purified gyroxin showed esterase and neurotoxic activities. In the radiocromatographic profile obtained from Sephadex G.100 column the radioiodination mixture was fractionated into five peaks. The radioeletrophoretic assay identified the [$^{125}$I]gyroxin in fraction 4 and the purity was estimated in 85.4% (Fig. 1).

The first way used to analyze the biodistribution data compares the total dose percentage incorporated to the organ with the body mass (%Dose/g) in a specific time interval (Fig. 2). This kind of analysis facilitates the visualization of the [$^{125}$I]gyroxin pathway during the DME process. The second method used presents the kinetic profile in each organ and allows the identification of the organs that accumulate [$^{125}$I]gyroxin (Figs 3 and 4). Another approach is to correlate the data obtained with [$^{125}$I]gyroxin in different organs with the tissue distribution of the protease activated receptors (PARs)[12] (Table 1).

The data obtained in the biodistribution assay allowed grouping the organs into three. The first group: organs that followed the blood kinetics: lung, heart and brain. The second group: organs related to metabolisms and elimination: liver, kidney and intestine. The third group: organs in which the gyroxin concentration increased during the observation period: spleen, skeletal muscle and stomach and thyroid.

In the first hour the biggest %Dose/g occurred in the liver and kidney, decreasing afterwards. After one hour the [$^{125}$I]gyroxin concentration increased in the stomach. Analyzing the radioactive profile detected in the liver and kidney it suggested that [$^{125}$I]gyroxin had hepatic metabolism and renal excretion. The radioactivity found in plasma and total blood was similar.

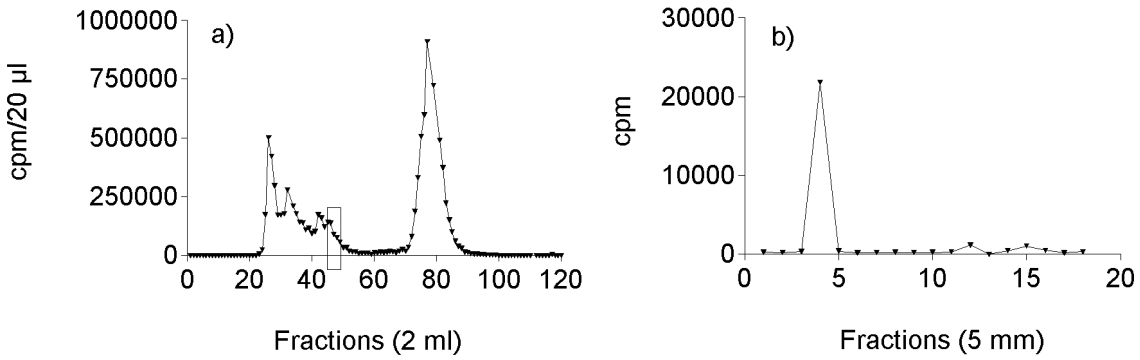


*Fig. 1.* (a) Chromatogram of labeling reaction mixture of gyroxin with $^{125}$I. Sephadex G.100 column (2.5×50 cm$^2$) eluted with 50mM sodium phosphate buffer pH 7.4 containing 0.1% of bovine serum albumin. Fractions of 2.0 ml and flow rate of 12 ml/h were used. The box indicate the fractions of [$^{125}$I]gyroxin chose for in vivo assay. (b) Electrophoretic analysis (SDS-PAGE 15%) of [$^{125}$I]gyroxin

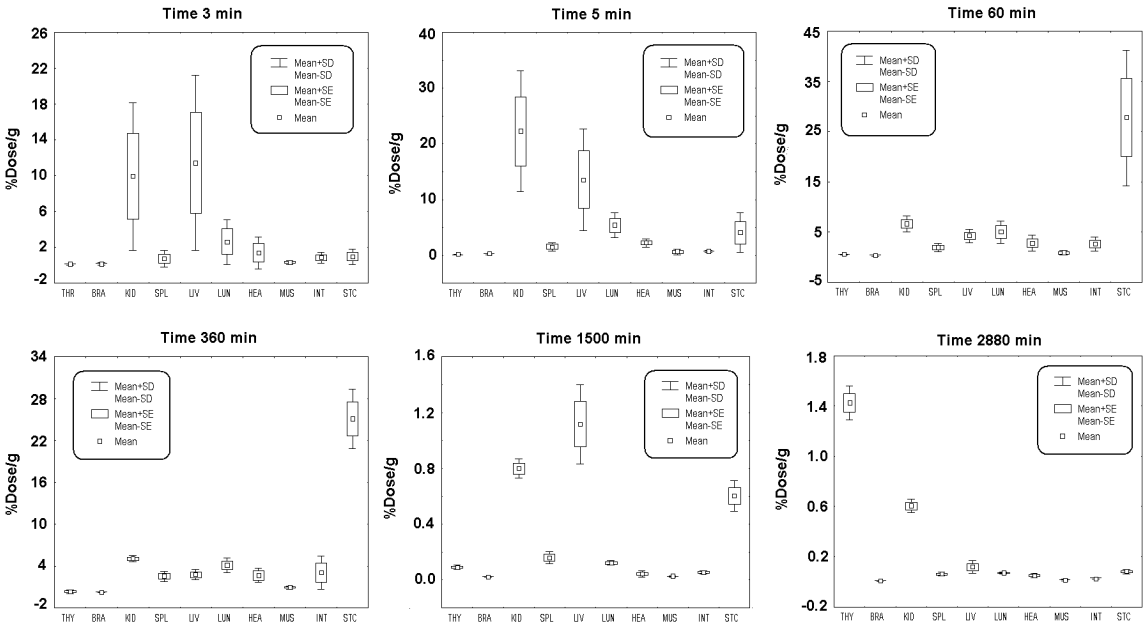


*Fig. 2.* [$^{125}$I]gyroxin detected in different organs THY, BRA, KID, SPL, LIV, LUN, HEA, MUS, INT and STC (thyroid, brain, kidney, spleen, liver, lung, heart , muscle, intestine, and stomach, respectively) in each time interval of 3, 5, 60, 360, 1500 and 2880 minutes. The measured activities were expressed as means and standard deviations and standard errors

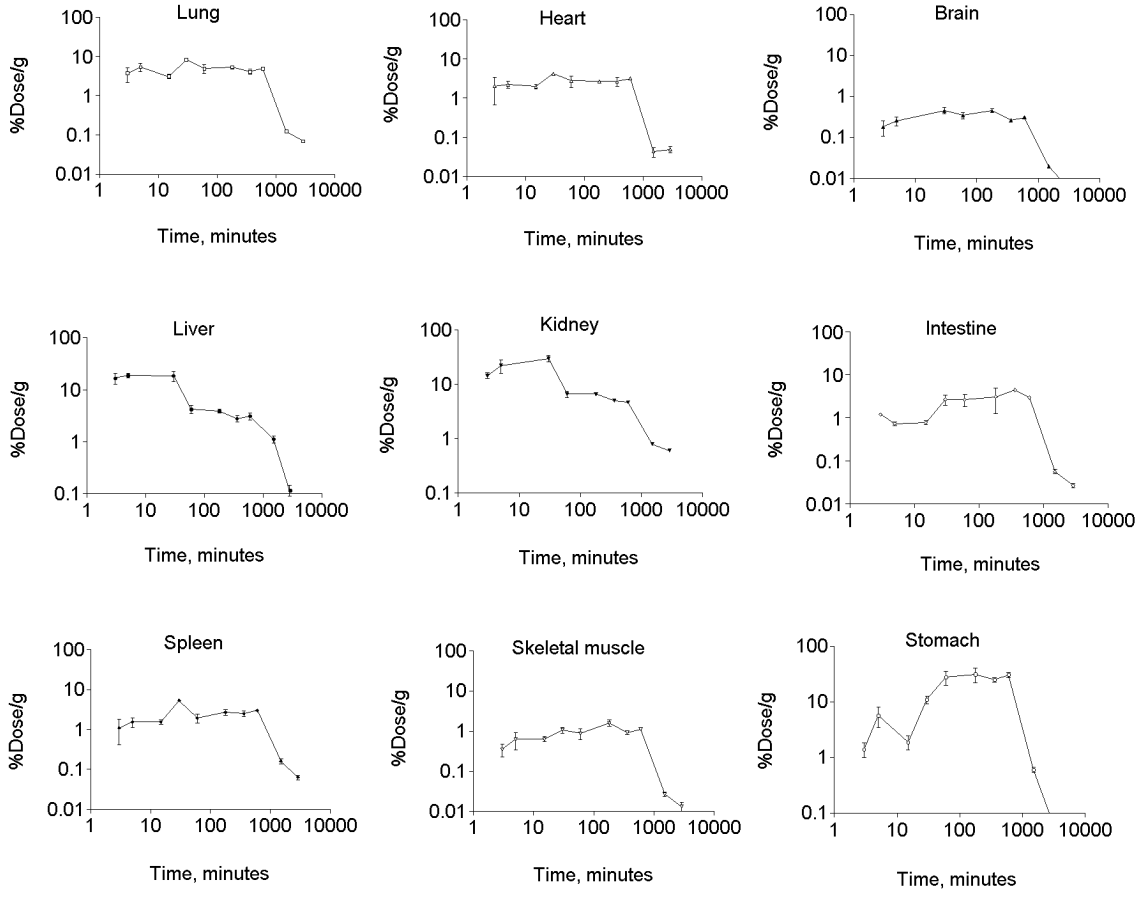


*Fig. 3.* [$^{125}$I]gyroxin kinetic behavior in each organs over 48 hours after intravenous administration. Data were expressed as the percentage of total dose injected per tissue weight (%Dose/g). The kinetic behavior allowed differentiating the organs in three groups. The first group with lung, heart and brain; the second, liver, kidney and intestine and finally, the third, with spleen, skeletal muscle and stomach

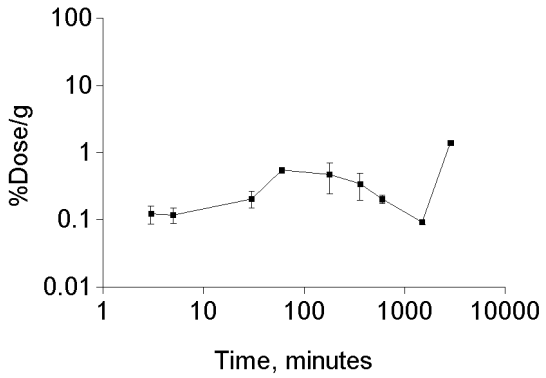


*Fig. 4.* Kinetic of thyroid gland captation of free $^{125}$I

*Table 1.* [$^{125}$I]gyroxin distribution and its correlation with proteinase activated receptors localization in various organs[12]

| Group | PAR-1 | PAR-2 | PAR-3 | PAR-4 |
|---|---|---|---|---|
| 1 | Brain<br>Heart<br>Lung | | Heart | Lung |
| 2 | Kidney | Kidney<br>Intestine<br>Liver | Intestine<br>Kidney | Intestine |
| 3 | Stomach | | Stomach | Skeletal muscle |

Low quantities of radioactivity were found in the brain, about 0.25 to 0.5% from the total injected dose and this percentage seemed to be stable during the whole time interval observed, similarly to the blood kinetic. This can indicate that gyroxin has no selectivity for the brain as was already related by CAMILLO[10] who suggested that gyroxin has a low capacity to pass through the blood brain barrier and that the neurotoxical effects caused by its injections should have been promoted by an intermediate factor.

The thyroid uptake is commonly assumed as an indicator of radioiodinated compounds stability in in vivo assays.[8,13,14] This gland actively uptake free iodine. The increasing in thyroid concentration of iodine is a good indicator of the [$^{125}$I]gyroxin deiodination. The absence of radioactivity in the thyroid confirmed the purity of the tracer and suggested that the metabolism of the [$^{125}$I]gyroxin was very slow in this mice strain.

Some physiological activities of serino proteases, as trypsin, thrombin and thrombin-like, are mediated by protease activated receptors (PARs). Since gyroxin is a serine protease, its action mechanism could be correlated with these receptors. It is well known that by PARs activation, some serine proteases increase endothelial cells permeability to macromolecules.[15] These receptors were also described in mammal's brains.[16] Changes in blood brain barrier are common responses to various physiologic events, as pH and arterial blood pressure alterations, nutrients and oxygen supply decrease.[17] Based on information obtained from literature regarding PARs distribution it was assumed that they could be correlated with gyroxin neurotoxicity. Although no experimental test was carried out, this hypothesis was checked comparing the concentration of gyroxin obtained in this work with theoretical distribution of PARs.

The data obtained with [$^{125}$I]gyroxin allowed to detect the gyroxin presence in different organs and suggests that Group 1 was more correlated with PAR-1 than PAR-3 and PAR-4 while Group 2 was more correlated with PAR-2. Group 3 has had no significant correlation.

The PAR-2 and PAR-1, to a certain extent (as it is present in kidney and stomach) are abundant in the gastrointestinal system what may indicate a correlation between the higher gyroxin concentration and these PARs presence in this system. The progressive accumulation in the stomach could be related to PAR-1 and PAR-3 presence in this organ.

This study has made the first correlation about gyroxin and its possible interaction with the proteases activated receptors present in different organs. Further experimental studies are necessary to characterize this interaction and its effects in the organisms.

## Conclusions

The methods used for gyroxin isolation and radioiodination were adequate, allowing to obtain a stable radiotracer with high purity for in vivo assays. The radiotracer allowed determining the gyroxin distribution in the experimental organism. The organs were divided into three groups and related to PARs presence. The data also suggested a hepatic metabolism and renal elimination.

*

The authors are grateful to Dr. Paulo Sérgio CARDOSO DA SILVA for helpful suggestion on graphical analysis. This research was supported by CAPES.

## References

1. M. GIBALDI, Biopharmaceuticals and Clinical Pharmacokinetics, Lea & Febiger, Philadelphia, 1991.
2. A. BARRIO, Acta Physiol. Lat., 11 (1961) 224.
3. H. BARRABIN, J. L. MARTIARENA, J. C. VIDAL, A. BARRIO, in: Toxins: Animals, Plant and Microbial, P. ROSEMBERG (Ed.), Pergamon Press, New York, 1978, p. 113.
4. I. RAW, M. C. ROCHA, M. I. ESTEVES, A. S. KAMIGUTI, Braz. J. Med. Biol. Res., 19 (1986) 333.
5. F. S. MARKLAND, Toxicon, 36 (1998) 1749.
6. Y. S. KOH, K. H. CHUNG, D. S. KIM, Toxicon, 39 (2001) 555.

7. F. BUCARETCHI, S. R. F. HERRERA, S. HYSLOP, E. C. E. BACARAT, R. J. VIEIRA, Rev. Instr. Med. Trop., 44 (2002) 133.
8. TH. M. BEHR, M. GOTTHARDT, W. BECKER, M. BÉHÉ, Nuklearmedizin, 41 (2002) 71.
9. M. AP. CAMILLO, P. C. ARRUDA PAES, L. R. P.TRONCONE, J. R. ROGERO, Toxicon, 39 (2001) 843.
10. M. A. P CAMILLO, P. C. ARRUDA PAES, M. T. C. P. RIBELA, J. R. ROGERO, in: 4th Meeting on Nuclear Applications, Poços de Caldas, Brazil, 1997.
11. U. K. LAEMMLI, Nature, 227 (1970) 680.
12. A. KAWABATA, R. KURODA, Japan J. Pharmacol., 82 (2000) 171.
13. K. KAWAI, Y. FUJIBAYASHI, H. SAJI, J. KONISHI, A. KUBODERA, A. YOKOYAMA, Nucl. Med. Biol., 17 (1990) 369.
14. S. H. SONG, K. H. JUNG, J. Y. PAIK, B. H. KOH, J. S. BAE, Y. S. CHOE, K. H. LEE, B. T. KIM, Nucl. Med. Biol., 32 (2005) 845.
15. L. T. NGUYEN, H. LUM, C. TERUPPATHI, A. B. MALIK, Am. J. Physiol.-Cell. Physiol., 273 (1997) 1756.
16. S. R. MACFARLANE, M. J. SEATTER, T. KANKE, G. D. HUNTER, R. PLEVIN, Pharmacol. Rev., 53 (2001) 245.
17. M. YEPES, M. SANDKVIST, E. G. MOORE, T. H. BUGGE, D. K. STRICKLAND, D. A. LAWRENCE, J. Clin. Invest., 112 (2003) 1533.