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TOXICOKINETIC STUDY OF A SEA ANEMONE TOXIN IN MICE AFTER INTRAPERITONEAL INJECTION

Inas S. Kamal*, Abdalla E. Ali and Mohamed N. Gomaa*****

** Narcotics, Poisons and Ergogenic aids Dep. and *** Marine Toxins Lab., National Research Center and ** Dept. of Marine Science Fac. of Science, Suez Canal Univ.*

ABSTRACT

One of the two low molecular weight (200-300 Dalton) toxins extracted from sea anemone, *Gyrostoma helianthus*, from the vicinity of Hurgada, Red Sea, Egypt was subjected to a toxicokinetic study. The concentration–time profile of the acetylcholine esterase (AchE) inhibitor sea anemone toxin was characterized using 20g male mice injected with 10 mg toxin as a single dose. Serum and five tissues were collected at 8 interval points (5, 10, 20, 30, 45, 60, 75 and 90 minutes). The toxin was extracted from these biological matrixes by acetonitrile. The *Gyrostoma helianthus* toxin showed short action as the half–life values vary from 4.5 to 27.5 min .The maximum time (t_{max}) was 30 min in serum and in all tissues except the heart which peaked at 45 min. The sea anemone toxin also showed first order kinetic.

Keywords: Toxicokinetic - Sea Anemone Toxin - *Gyrostoma helianthus* -

INTRODUCTION

The study of marine organisms as a source of biologically active compounds is considered a very productive field, having already led to the discovery of various new pharmacological tools and medicines (Bhakuni, 1994; Munro *et al.*, 1999; Harvey, 2000 and Faulkner, 2001).

The work of Bergman and Feeney at the beginning of the 1950s initiated the study of marine natural products, and in the last few decades, an appreciable number of new compounds have been isolated from marine organisms (Bhakuni, 1994; Faulkner, 2000 a, b and Faulkner, 2001).

Marine life forms compared with terrestrial ones are expected to produce totally new secondary metabolite molecules that may exhibit neuroactivity. Moreover, the Red Sea environment, is one of the newly working areas that is considered being unique in its life forms (Gomaa *et al.*, 2000).

Sea anemones are predatory animals, they live attached to firm objects in the seas, usually the sea floor, rock or coral but they can slide around very slowly. Sea anemones are classified in the *Actinarians* which have generally column-shaped bodies with the mouth at one end, and the pedal disk (a muscular organ for attachment to substrates) at the other. Sea anemone had neurotoxic effect that paralyses small marine animals with stinging cells in the tentacles called nematocysts. This mechanism is one of the fastest actions in the animal kingdom (Paton, 1995).

LD_{50} of the most active sea anemone toxins and the most active scorbion toxins are similar in mice, and sea anemone toxin at high concentration prevents binding of scorpion toxins to their receptor. However, scorpion toxins have affinities for the Na^+ channel which are approximately 60 times higher than those found for the most active sea anemone toxins (Schweitz *et al.*, 1981).

The toxins of several species of sea anemone have different actions that were reported in previous studies (Paralysis to crabs and mammals (Beress and Beress, 1971); cytolytic and lethal effects of equinatoxin isolated from the sea anemone *Actinia equine L. (*Ferlan and Lebez, 1974 and 1976), ichthyotoxocity (Mebs *et al.*, 1983); hemolysis by caritoxin I, II, and III isolated from sea anemone *Actiniria cari* (Devlin, 1974; Macek *et al.*, 1982; Mebs and Gebauer, 1980) and protinase inhibition (Mebs *et al.*, 1983).

The neurotoxin of the sea anemone *Radianthus macrodactylus* produces pronounced cardiac stimulatory activity, inducing a positive inotropic effect in mammalians heart preparations (Alsen, 1983). Also, *Radianthus* neurotoxins slowes or blockes the inactivation process of the voltage dependent sodium channels (Sorokina *et al.,* 1984; Schwetiz *et al.*, 1985).

***Gyrostoma helianthus* neurotoxins delayed the activation of K^+ channel and showed reversible acetylcholine esterase inhibition in both brain and blood of mice (Gomaa and Aboul-Enein, 2000). Reversible acetylcholine esterase (Ach E) inhibition activity plays an important role as a safe insecticide. This gives hope to try the use of this toxin as an insecticide.**

The present work was designed to study the toxicokinetics of *Gyrostoma heleanthus* AchE inhibitor (GHT) toxin in mice in order to understand its reversible physiological activity .

MATERIALS AND METHODS

1. Sample Collections

The toxin was extracted from sea anemone, *Gyrostoma helianthus*, collected from the vicinity of Hurghada, Red sea, Egypt. The animals were collected by trained drivers from areas famous to harbor the specific species under study. Samples were kept in seawater during the trip then washed with distilled water in the laboratories of Hurghada marine station, National Institute of Oceanography and Fishery. Collected specimens were centrifuged to remove excess water and the animal net weight was recorded.

2. Extraction

The bioactive material was extracted following the technique reported by Gomaa *et al.* (2000). Intact washed sea anemone samples were blended with equal volume of absolute ethanol (1:1 w/v) for 3 minutes, and then centrifuged at 2570 g for 10 minutes. After removing the organic layer, the extraction was repeated. A third extraction was done with 50% aqueous ethanol, and the collected supernatant was mixed. Defatting and partial depigmentation of the supernatant was done with equal volume of n-hexane for three successive times, then the aqueous ethanolic extract was evaporated under vacuum and the remained aqueous portions were kept for further purification.

3. Isolation and purification

Fractionation and purification of the crude extract was carried out using different techniques following the bioassay guided fractionation protocol reported by Gomaa and Aboul-Enein (2000), i.e. ultrafilteration, Bio Gel P2 gel filtration and with the use of high performance liquid chromatography (HPLC).

a. Ultrafiltration

Aqueous crude extract was filtered through molecular weight exclusion membrane filters with cut off 10, 5, 3, 1, and 0.5 k Dalton (76 mm in diameter, Millipore Corporation, Bedford, MA). Ultrafiltration was performed under pressure of nitrogen gas (40 $Kg.cm^{-2}$). The filtrate (less than 500 Dalton) was lyophilized and the net dry weight was determined (Gomaa and Aboul-Enein, 2000).

b. BioGel P2 gel filtration

Gel filtration column chromatography was prepared using a 3.5 X 80 cm Omni LC column, packed with BioGel P2 (BioRad Laboratories, Richmond, CA), to reach a bed height of 75 cm and a bed volume of 728 ml (Shimizu *et al.*, 1975; Buckley *et al.*, 1975; Hall, 1982; Gomaa, 1990). Fifteen ml of Milli Q water was used to redissolve 5g of the less than 0.5 k Dalton freeze-dried filtrate and then applied on the top of the BioGel P2 column. The column was eluted with 2 bed volumes of Milli Q water using a peristaltic pump to provide a flow rate of 48 $ml.hr^{-1}$, and 5-ml fraction was collected.

Buckley spot plate technique (Buckley *et al.*, 1975) was used to detect fluorescence and quenching activity of the collected fractions along with their reaction with ninhydrin. Aliquots of all the collected fractions, 5 µl each, were spotted on 10x10 cm silica gel TLC plates (precoated, type 60 F254, with fluorescent indicator, aluminum backed, E. Merck). Fluorescent and quenching activity was observed under long wave (366 nm) and short wave (254 nm) UV. Spot plates were sprayed with 1% ninhydrin in ethanol to detect the presence of the free α-amino groups (purple color) or the substituted α-amino group (yellow color) in the toxic fractions.

Toxic fractions, detected by mouse assay, were compared with any physical property that may appear by Buckley spot test. The percent bed volume was calculated for each fraction to correlate between the positions of the toxic fractions with the calculated percent bed volume (Gomaa and Aboul-Enein, 2000). *Invitro* AchE inhibition test (Ellman *et al.*, 1961) was used to confirm the mode of action of the collected toxic fractions.

c. HPLC

HPLC was used to purify the collected toxic fraction proved to inhibit the AchE activity. The HPLC conditions used was according to Gomaa and Abu Elenien, (2000). The HPLC column used was spheri 5 silica 5µm 100x4.6 mm (Applied biosystems Inc., Hoster City, A 94404 USA, Brownlee columns). The mobile phase system (H_2O : MeOH : Acetonitrile) was used in a gradient program as shown in Table (1) and the flow rate was 1ml/min. The wavelength of the UV detector was set at 254nm.

Table (1): Gradient program of the mobile phase to purify the toxin compound causing acetylcholine esterase enzyme inhibition.

Time (min).	% water	% Methanol	% Acetonitrile
0	5	10	85
5	15	5	80
10	30	0	70
15	5	10	85

4. Toxicokinetic studies

a. Animals and doses

Albino Swiss male mice with average weight of 18-22g were used for the toxicokinetic study. Mice were obtained from Animal House Division, National Research Center, Cairo, Egypt. Three mice were used at each time point. The mice were injected intraperitoneally with single dose of 10 mg/20 g. The dose of sea anemone, *Gyrostoma helianthus* toxin used in this study was chosen based on the previous study of the mouse unit done by Gomaa *et al.* (2000).

b. Blood and tissues samples

Blood and tissues samples were taken at time intervals of 5, 10, 20, 30, 45, 60, 75 and 90 minutes. Blood samples were taken via the orbital sinuses and serum was separated by centrifugation at 2310 g for 15 minutes. Collected tissue samples (Brain, liver, spleen, kidneys and heart) were rinsed with saline, and weighed. Both serum and organs were stored at -20°C until toxin extracted and analyzed.

c. Sea anemone toxin extraction from biological samples

The toxin was extracted from serum by adding 3ml acetonitrile to 100 µl serum sample, then mixed for few seconds using vortex. Tissues were homogenized after adding acetonitrile to tissue samples in a ratio of 2:1 (v:w), then centrifuged at 2310 x g for 10 minutes. After separating the supernatant, extraction was repeated twice in both serum and tissues. The collected supernatant was evaporated to dryness under a nitrogen stream (Moffat *et al.* 2004). The dried film was dissolved in 500 µl mobile phase. Samples were filtered through a 0.45 µ membrane filter to be prepared for HPLC analysis (Gomaa and Aboul-Enein, 2000).

d. HPLC analysis for toxicokinetic determination

The same HPLC conditions as previously mentioned in the purification step (3.C) were used in the determination of relevant toxicokinetic. To ensure that no interferences due to serum or tissues components exist in the HPLC chromatogram, sea anemone toxin (GHT) along with toxin-free mice serum and tissues extracts were observed in the HPLC chromatograms.

e. Recovery of extraction method

The extraction method recovery was assessed using the HPLC. A calibration curve was analyzed at five different concentrations of sea anemone toxin (GHT) (2.3, 3.4, 4.6, 5.8, 6.9 mg/ml) dissolved in mobile phase. Spiked blank mice sera standards were prepared at the same five concentrations. Toxins were extracted by acetonitrile. The percent recovery of toxin in mice sera was calculated against the HPLC values of the isolated pure GHT of the same concentrations dissolved in the mobile phase.

f. Statistical analysis

The serum and tissues concentrations versus time profiles for sea anemone toxin were calculated as means and standard deviations using SPSS computer software version 7.5.

g. Toxicokinetic Analysis

Area under the curve (AUC) verrus time values was calculated for the averaged data with the log-trapezoidal rule from time zero to the last experimental data point. AUC values were then extrapolated from the last experimental data point to infinity by calculating C/λ_z where C is the predicted concentration at the last measured time point, and λ_z is the terminal rate constant. The terminal half–life ($t_{1/2}$) was estimated with data from the terminal elimination phase.

$$t_{1/2} = (0.693)/ K_{el}$$

where K_{el} was apparent elimination rate constant

Volume of distribution (V_d) is estimated according to the following equation:

$$V_d = dose/C_{p0}$$

Where: V_d is the volume of distribution

C_{p0} predicted maximum concentration

RESULTS AND DISCUSSIONS

Toxin profile by HPLC

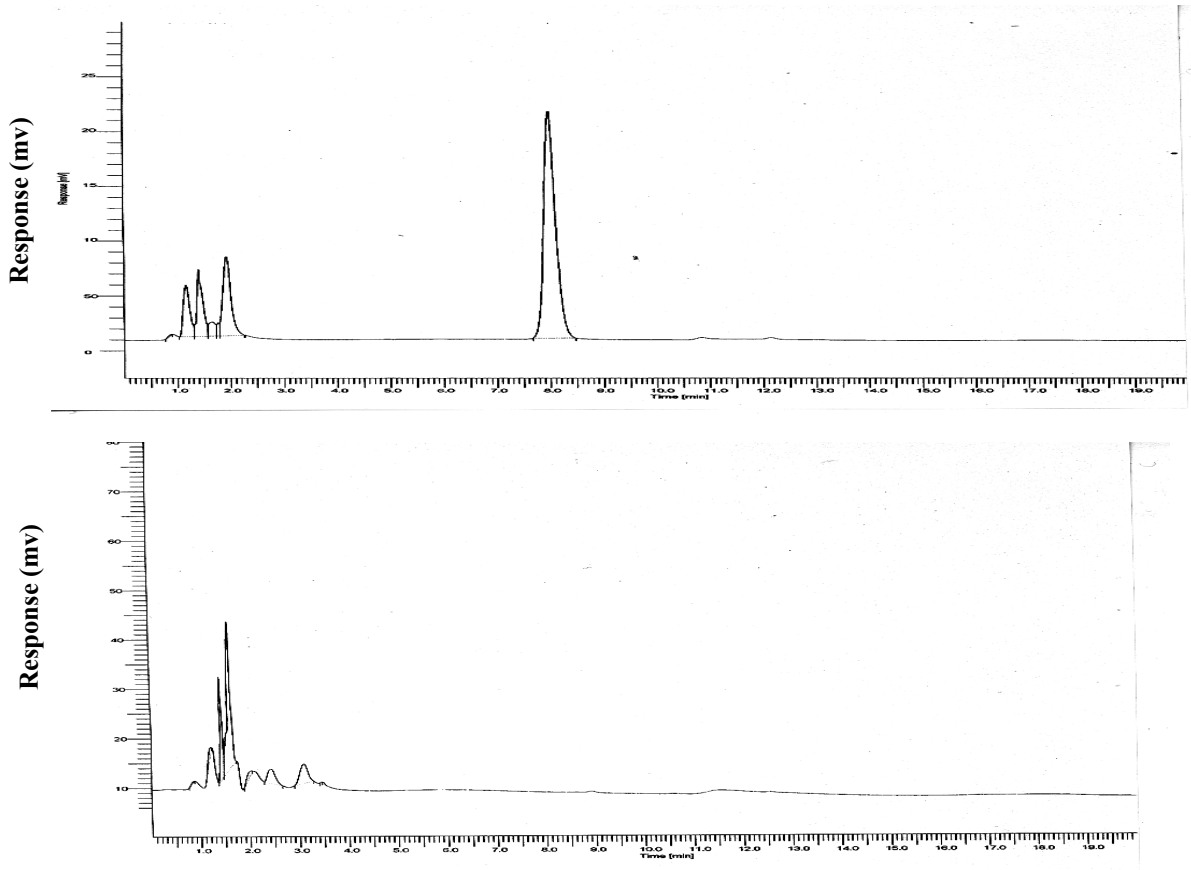
Comparing the HPLC chromatograms of the sea anemone toxin (GHT) and toxin-free mouse serum (Fig. 1) showed that the area of the chromatogram where the toxin was observed (Fig. 1a) was clear of any interference in the mouse serum chromatogram (Fig 1b). Same observations were also noticed in chromatograms of the different mice tissues.

Extraction method recovery of GHT

The extraction method recovery was assessed using the HPLC determination of different GHT concentration (2.3, 3.4, 4.6, 5.8, 6.9 mg/ml) dissolved in mouse serum matrix and extracted by acetonitrile. The same concentrations of the isolated pure GHT dissolved in the mobile phase matrix were also determined by HPLC. Plotting the toxin concentration of both matrixes against the HPLC-UV response (in mv) resulted in two straight lines almost matched at low concentrations and separated away from each other at higher concentrations (Fig. 2). This means that the recovery of the toxin from the serum is decreased in higher concentrations. The overall percent recovery (93.7%) was calculated by dividing the slope value of the toxin in serum (3.71) over the slope value of the toxin in mobile phase (3.96).

Toxicokinetic of GHT in serum and tissues after i.p. injection

HPLC was used to trace the toxicokinetic of the sea anemone toxin (GHT) in mouse serum and tissues. The complete reversibility of the toxin that was reported by Gomaa *et al.* (2000) was observed and confirmed by tracing the toxin kinetic by HPLC (Fig. 3). After 5 minutes of i.p. injection the toxin concentration detected in the serum was 0.095 mg/ml followed by slight increase (0.19 mg/ml) after 15 minutes. A sudden increase in the toxin concentration in serum (1.71 mg/ml) was detected after 30 minutes recording the maximum concentration among all time intervals.


Response (mv)
Response (mv)
Response (AU)
Toxin concentration (μg/ml)

The toxin concentration showed a decrease after 60 minutes (0.95 mg/ml) and the lowest concentration (0.048 mg/ml) was detected after 90 minutes. No toxin concentration was detected in the serum after 120 minutes. This type of toxin profile in the serum that was observed by HPLC in the current study, was also reported by Gomaa *et al.* (2000) using *in vivo* study of AchE activity. They reported that maximum inhibition of AchE activity in serum samples was recorded 30 minutes after i.p. injection and after 120 minutes the enzyme activity restored its original level.

The time course of GHT toxin concentrations in serum demonstrated a typical semilogarithmic plot of drug concentration in serum following intraperitoneally administration. The sea anemone toxin was rapidly absorbed and reached a peak C_{max} (1711.8 µg/ml) in mice serum after 30 min. No toxin was detected in both serum and tissues after 120 minutes (Fig. 4). This agreed with Gibaldi (1984) who stated that the faster a drug is absorbed, the higher is the maximum concentration in plasma after a given dose, and the shorter is the to reach the peak is observed. This also confirmed the previous study of Gomaa *et al.*, (2000) where the maximum of Ach E inhibition by GHT was obtained after 30 min in both mice serum and brain and enzyme activity was restored after 120 min.

This toxin showed short half life $t_{1/2}$ (Table 2) in serum (16.5 min) and low volume of distribution V_d (0.07 L/Kg). After intraperitoneal route of administration absorption is facilitated by the large surface area of the peritoneal cavity. The chemical mainly enters the liver by the portal circulation; thus, under goes first pass metabolism (Blaschke and Rubin, 1979; Wilkinson, 1986) which reduced the action of the parent drug (Moffat, 2004).

The AUC for serum was 94.76 mg/ml/min and the constant elimination rate (K_{el}) was 0.042 min^{-1}. We found that the toxin elimination followed first order kinetic as the rate of removal is proportional to the concentration remaining and so a constant fraction of the toxin is excreted at any given time (Gibaldi, 1984 and Timbrell, 2000).

Our results regarding toxin concentration in different tissues (Fig 4) showed that the highest toxin concentration was in the kidney with C_{max} (86.3 µg/g) followed by the spleen (79.0 µg/g), liver (24.1 µg/g) and brain (17.7 µg/g) and the lowest C_{max} was in the heart (12.4 µg/g). Also the half-life ($t_{1/2}$) in tissues (Table 2) was the lowest in the kidney (4.5 minutes) followed by the spleen (10.0 minutes), heart (12.5 minutes) and brain (22.0 minutes) and the highest was in the liver (27.5 minutes). The AUC for tissues as shown in Table (2) was the highest in the kidney (3.65 mg/ml/min) followed by the spleen (2.86 mg/ml/min), liver (1.84 mg/ml/min) and brain (0.96 mg/ml/min) and the lowest was in the heart (0.30 mg/ml/min).

Table (2): Toxicokinetic parameters of sea anemone, *Gyrostoma helianthus* in mice after 10 mg/20 g i.p. injection.

Samples	$t_{1/2}$ min	AUC Mg/ml/min	C_{max}
Serum	16.5	94.76	1711.8 µg/ml
Kidney	4.5	3.65	86.3 µg/g
Spleen	10.0	2.86	79.0 µg/g
Liver	27.5	1.84	24.1 µg/g
Brain	22.0	0.96	17.7 µg/g
Heart	12.5	0.30	12.4 µg/g

AUC:Area under the toxin concentration curve versus time.
$t_{1/2}$: half life of the toxin.
C_{max}: Peak concentration.

The tissue:serum ratios illustrated in Fig (5) showed that tissue/serum sea anemone toxin (GHT) didn't reach the steady–state. The decreased concentrations in all tissues below serum level indicated that it didn't distribute extensively into these tissues (Table 3).

The kidney is supposed to be the major excretory organ that can efficiently remove only highly hydrophilic, usually ionized chemicals (Guengerich and Liebler, 1985; Pirmohamed *et al.*, 1994 ; Klaassen and Watkins, 1999).

The spleen is one of the lymphoid organs that filter the blood by removing both foreign antigens, and circulating dead cells and cellular components, and thus act as biological sieve (Klaassen and Watkins, 1999). This may the finding explain that the spleen toxin concentration and spleen:serum ratio was higher than liver, brain and heart. Further investigations are therefore, needed to determine the effect of sea anemone toxin (GHT) on the immune system.

Table (3): Tissue:Serum ratio of the sea anemone, *Gyrostoma helianthus* toxin in mice after 10 mg/20 g intraperitoneal injection.

Time (min)	Liver	Brain	Kidney	Spleen	Heart
5	0.008	0.015	0.025	0.022	0.0
10	0.026	0.011	0.058	0.030	0.0
20	0.014	0.008	0.036	0.039	0.003
30	0.013	0.010	0.048	0.048	0.007
45	0.016	0.015	0.058	0.031	0.016
60	0.017	0.019	0.052	0.037	0.003
75	0.045	0.025	0.062	0.059	0.006
90	0.050	0.019	0.024	0.014	0.003

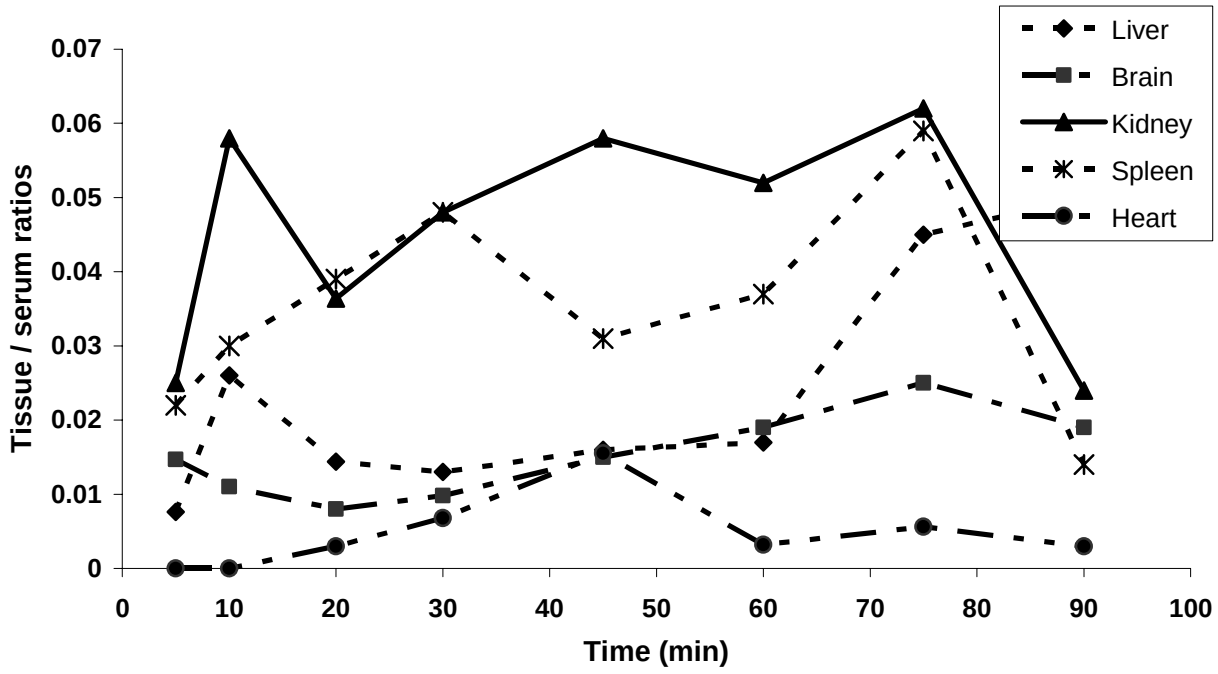


Fig 5. Tissue serum ratio of sea anemone *Gyrostoma helianthus* toxins following a 10mg/20g i.p. dose in mice (n=3 mice per time point)

Sea anemone toxin (GHT) brain:serum ratio was low. This low ratio was expected as this toxin is hydrophilic to (Gomaa *et al.*, 2000). Increased lipid solubility enhances the rate of penetration of the toxin into CNS through the blood-brain barrier, whereas ionization greatly diminishes it (Klaassen and Watkins, 1999). This result conformed to that of Gomaa *et al.* (2000), who reported that the maximum inhibition of brain acetylcholine esterase enzyme (24.2%) was lower than the maximum inhibition of serum choline esterase enzyme (87.1%).

Conclusion:

The present study revealed that sea anemone toxin (GHT) had short half life $t_{1/2}$ (16.5 min) and low V_d (0.07 L/Kg). All tissue:serum ratios didn't reach the steady state and the toxin was not equally distributed to all of the tissues examined so it had no target organ to store in it. Elimination of sea anemone toxin (GHT) was mainly by the kidney then the liver, and it followed first order kinetic.

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