In vivo acute toxicity assessment of a novel quinoxalinone (6-nitro-2 (1H)-quinoxalinone) in Wistar rats

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Abstract: The quinoxaline derivatives are an important class of heterocyclic compounds, obtained from chemical azote replacement of carbone atom. Fusion of quinoxaline production is relatively easy as they are obviously synthesized by the fusion of two aromatic rings, benzene and pyrazine. The new quinoxalinique derivative, 6-nitro-2 (1H)-quinoxalinone (NQX), has been synthesized in our laboratory. However, the related toxic effect on rat remains unknown. The present work aims to study the acute toxicity of NQX in normal Wistar rats. Seven groups of female rats received an intraperitoneal (i.p.) injection of 0 (control), 20, 40, 60, 120, 200 and 300 mg/kg of the NQX and followed for 14 days. Mortalities, behavioural changes, weight, changes in food and water uptake, urine output and weight of faeces were monitored. At the end of the experiment, the rats receiving the no-observed-adverse-effect level (NOAEL) are sacrificed, blood and organs were collected and haematological and biochemical parameters were analysed in sera sample. The results showed that the NQX Lethal Dose 50 (LD50) was 161.16 mg/kg. The administration of NQX at a dose of 40 mg/kg (NOAEL dose) did not affect animal viability and body weight. In addition, food intake, water intake and urine output remain unchanged. Furthermore, at the NOAEL dose, the levels of blood cells (erythrocytes and leukocytes), haemoglobin, biochemical parameters (glucose, cholesterol, triglycerides, urea, creatinine, bilirubin, total protein and transaminase) and organ's weights were determined.
(liver, kidney, spleen, pancreas, heart and brain) were not affected. NQX seems to be relatively saved at the dose of 40 mg/kg in normal Wistar rats and could possibly be tested after further analysis in a preliminary clinical test.

**Subjects:** Pharmaceutical Science; Drug Discovery; Toxicology

**Keywords:** 6-nitro-2 (1H)-quinoxalinone; LD50; NOAEL; acute toxicity; Wistar rat

1. Introduction

Quinoxaline or benzopyrazine is a heterocyclic compound containing a benzene and pyrazine ring. Pyrazines are colourless compounds, which are stable and possess good water solubility. Unlike pyridines, they are expensive, hardly available and are therefore rarely used as the starting material for the synthesis of their derivatives. Diazines are bonded to the benzene ring to create quinoxaline.

The quinoxaline derivatives are an important class of heterocyclic compounds, in which N replaces one or more carbon atoms of the naphthalene ring (Deepika et al., 2011). The main location to the quinoxaline loop system is shown in Figure 1, whereas 2 and 3 are placed at alpha-positions (Chesseman & Cookson, 1979).

Quinoxaline derivatives play an important role in pharmaceutical industry. These products are largely used as metal corrosion inhibitor (El Adnani et al., 2012; Kabanda & Ebenso, 2012), the electroluminescent materials process (Carta et al., 2002; Chang et al., 2012; Justin Thomas et al., 2005; Sharma, Raisinghani, Abraham, Pardasani, & Mukherjee, 2009) and are key elements in the chemical synthesis of the porphyrins.

Due to their wide range of biological properties, they are of great importance in pharmaceutical industry (Carta et al., 2002; Deepika et al., 2011; Kulkarni, Revankar, Kirasur, & Hugar, 2012). In addition, quinoxalin backbone is also used as an intermediate in the design of new quinoxalinone derivatives which can be used as an antidepressant (Nakache et al., 2012; Sarges et al., 1990), anxiolytic (Nakache et al., 2012; Olayiwola, Obafemi, & Taiwo, 2007), antagonists of 5-HT3 receptors (Mahesh, Devadoss, Pandey, Bhatt, & Yadav, 2010), antagonists of the glycine receptor/NMDA (Varano et al., 2001), inhibitor of MAO-A (Hassan, Khattab, Bekhit, & Amer, 2006) and anti-convulsion in epilepsy animal models (Löscher & Hönack, 1994; Nordholm, Sheardown, & Honoré, 1997).

The common procedure for their synthesis consists of chemical condensation of o-disubstituted benzene with a two-carbon synthon. Therefore, the chemical reaction of o-phenylenediamine with α-dicarbonyl compounds leads to quinoxaline formation (Chesseman & Cookson, 1979) (Figure 2).

Many approaches are available and well known for the synthesis of quinoxalinic derivatives. In fact they are involving the condensation of 1,2-diamines with α-diketones (Brown, Taylor, & Ellman, 2004), 1,4-addition of 1,2-diamines to diazenylbutenes (Aparicio et al., 2006), cyclization-oxidation of phenacyl bromides (Kunkuma, Bethala, Bhongiri, Rachapudi, & Potharaju, 2011; Shi, Dou, Ni, Shi, & Li, 2008) and oxidative coupling of epoxides with ene-1,2-diamines (Antoniotti & Duñach, 2002).
The aim of this study was to evaluate the acute toxicity of NQX in Wistar rats. This animal model will allow us to assess the lethal minimum dose (LMD), the lethal dose 50 (LD50) and the No-Observed-Adverse-Effect Level (NOAEL). The effects of NQX at the NOAEL dose have been assessed following the evolution of haematological and biochemical parameters.

2. Materials and methods

2.1. Product and animals

NQX was synthesized in the “Laboratoire d’Agroressources et Génie des Procédés, Department of chemistry, Faculty of Sciences, Ibn Tofail University, Kenitra – Morocco” adopting the HONG protocol (Hong, Kim, Park, & Kim, 2000), which consists of condensing α-phenylenediamine with glyoxilic acid in refluxed n-butanol for 5 h (Figure 3). After solvent filtration, the rough was purified by recrystallization in ethanol and dimethylformamide (Nakache et al., 2012).

The study was conducted using a 3-month aged female Wistar rats. The experimental protocol is carried out according to the Organization for Economic Cooperation and Development (OECD) guidelines, the Food and Drug Administration (FDA) and the Canadian Council of Animal Care (CCAC) (Canadian Council on Animal Care, 1993; OCDE, 2001). The weight of the rats was in a range of ± 20% of the average weight of all animals at the beginning of the study. The animals were divided in separate metabolic cages and maintained at a temperature of 25–30°C and humidity between 60 and 70%. Animals had access to water and food (standard diet) ad libitum and were acclimation for at least five days before the start of the experiment.

2.2. Determination of LMD, LD50, NOAEL and observation of acute intoxication manifestations

Mortality after the administration of a single dose of NQX establishes the LMD that can kill at least one treated animal, the LD50 that kills 50% of treated animals and the NOAEL after 14 days. The OECD recommends administering the compound to several groups of animals and increases the doses to obtain 100% mortality. Doses administered were between 5 and 5 g/kg of the body weight allowing to test toxicity following Hodge Sterner standards (Claude, 1988; OCDE, 2000).

The study was performed on seven different groups of six rats each. The rats received an i.p. injection of the test compound (NQX) at different doses in constant volume regardless of the tested compound concentration (1 ml/per 200 g body weight).

The rats were divided into different groups:

- 1st, control group: Rats received an i.p. injection of 1-ml saline (10%DMSO + 0.9% NaCl) per 200 g of body weight.
- 2nd to 7th groups: Rats received a dose of 20, 40, 60, 120, 200 or 300 mg/kg diluted NQX in 1-ml vehicle solution (10%DMSO + 0.9% NaCl).

After i.p. injection, rats were placed in metabolic cage. Body weight, food and water uptake, faeces weight and urine volume output were monitored for 14 days. Furthermore, some physiological parameters such as heart and breathing rate were evaluated in addition to behaviour change and behavioural responses to external stimuli (e.g. noise, variation of light intensity).
We followed the mortality rate at different doses with all the behavioural changes, such as loss of balance, posture, scraping, appearance and fall of hairs, the appearance of faeces, diarrhoea, weight body change, aggression, bleeding, hypersalivation and the change in the intake of food and water and rectal temperature.

At the end of the experiment (day 14 later), the animals receiving the NOAEL dose were weighed and then anesthetized with sodium pentobarbital at 6.5% (0.1 ml per 100 g body weight) following a 12-h fasting. Blood is drawn by puncture of the abdominal aorta. One part of the blood has been collected in EDTA tubes for blood tests; the second part was collected in plain tubes for biochemical analysis. The organs (liver, spleen, heart and brain) were carefully removed, rinsed with 0.9‰ NaCl and then weighed.

2.3. Haematological and biochemical analysis

2.3.1. Haematology
The enumeration of erythrocytes and leukocytes has been done using Thomas cells and optical microscope at a magnification of 10 × 40. Haemoglobin was assayed by the colorimetric method of DRABKIN (cyanmethemoglobin).

2.3.2. Biochemical parameters
Renal function was assessed by measuring creatinine and urea in serum (Kits Sigma Chemical Company, St. Louis, MO, US). Liver function was assessed by the enzymatic activity of glutamic oxaloacetic transaminase (GOT) and glutamino-pyruvic (TGP) as well as the determination of bilirubin and total serum protein (Kits Sigma Chemical Company, St. Louis, MO, USA). Glucose, total cholesterol and serum triglycerides were evaluated using enzymatic methods (kits Sigma Chemical Company, St. Louis, MO, USA).

2.4. Statistical analysis
Results are presented as mean ± standard deviation. Multiple comparisons were performed using the ANOVA test. The differences are significant if $p < 0.05$.

3. Results

3.1. Assessment of acute toxicity
After i.p. injection of NQX at the dose of 20, 40, 60, 120, 200 and 300 mg/kg of the body weight, the rats were monitored for 14 days. The mortality rate was noted for each used dose and the dose-lethal graph was made.

Our results showed that the LMD of the NQX tested is 60 mg/kg, whereas the LD is 120 mg/kg (dose causing 33.33% death) and 200 mg/kg (dose causing 66.66% death) (Table 1, Figure 4). The LD50 of the NQX is therefore estimated at 161.16 mg/kg. All the rats receiving the NQX at the dose of 40 mg/kg survive to the administration, which leads us to suggest that this dose is the NOAEL dose. The administration of NQX induces several clinical signs that increase in severity and intensity proportionally to the administrated dose. The most characteristic signs include, decrease in motor activity, respiratory malfunctioning polypnea type, twists with hair straightening, agitation followed by sleepiness and diarrhoeal signs.

Toxicity signs occurred 24 h after the injection of the NQX, except for rats treated with a dose of 20 and 40 mg/kg, which have no noticeable clinical signs.

Our results show that the injection of the NQX at a dose of 40 mg/kg is the threshold at which both physiological and behaviour pathological states begin to appear in rats. From this dose, the disruption of physical appearance, fur, breathing, secretions, behaviour and temperature is becoming
stronger and shows a clear sign of toxicity. At a dose of 300 mg/kg, all animals are dying immediately after the injection, which can be considered as the endpoint from toxicology tests.

### 3.2. Changes in body weight, ingestions and excretions

Figure 5 depicts change in body weight following an i.p. injection of NQX. At the beginning of the experiment (d0), all group's weight was fluctuating between 200 and 260 g. The injection of the first two NQX doses (20 and 40 mg/kg) does not cause any significant changes in body weight evolution compared to controls at various times (D7 and D14). However, the injection of the other NQX doses (60, 120 and 200 mg/kg) induces a significant decrease in body weight in comparison to control group at D7 and D14. This effect was more pronounced at d14.

#### Table 1. Death rate after i.p. administration of NQX at different doses

| Dose (mg/kg) | Total deaths | Percentage of mortality (%) |
|--------------|--------------|-----------------------------|
| Group 1 (control): n = 6 | - | 0 | 0 |
| Group 2: n = 6 | 20 | 0 | 0 |
| Group 3: n = 6 | 40 | 0 | 0 |
| Group 4: n = 6 | 60 | 1 | 16.66 |
| Group 5: n = 6 | 120 | 2 | 33.33 |
| Group 6: n = 6 | 200 | 4 | 66.66 |
| Group 7: n = 6 | 300 | 6 | 100 |

Figure 4. Change in percentage of death after i.p. injection of NQX in normal Wistar rats.

Figure 5. Evolution of body weight in control and NQX-injected rats during 14 days.

Notes: Values are presented as mean ± SD. d0: primary weight; d7: weight after one week; d14: weight after 2 weeks. Dose 1: 20 mg/kg; dose 2: 40 mg/kg; dose 3: 60 mg/kg; dose 4: 120 mg/kg and dose 5 of 200 mg/kg.
Injecting 20 or 40 mg/kg of NQX did not cause any significant changes in the food and water consumption compared to controls during the first and second week of experiment. However, food ingestion (g/day/rat) and water consumption (ml/day/rat) were significantly reduced following the injection of higher doses of NQX (Figure 6).

The rats injected with 20 or 40 mg/kg of NQX did not cause any significant changes in faecal excretion expressed as (g/day/rat) and urine evacuation (ml/day/rat) compared to control during the 14 days of the study. As doses of the NQX increased, the faecal and urinary excretions were seriously reduced in treated rats compared to control (Figure 7).

### 3.3. Changes in haematological and biochemical parameters

The results show that NOAEL dose for NQX is 40 mg/kg. To confirm the non-toxicity effects of this dose, haematological and biochemical parameters were performed at day 14. 40 mg/kg of NQX did not cause any significant changes in leukocytes nor erythrocytes number compared to control (Table 2). Furthermore, this dose did not cause any significant changes in sera levels of glucose, total cholesterol, triglycerides, total protein, urea, creatinine and bilirubin as well as enzymatic activity of the GOT and TGP compared to control (Table 3).

### 3.4. Weight of various body organs

The relative weight of different organs (organ weight/animal weight × 100) indicates the relative weight changes of the organ to the body weight. Following the injection of 40 mg/kg of the NQX, the relative weight changes of different organs (liver, kidney, spleen, pancreas, heart and brain) in NQX-treated rats remained similar to controls (Table 4).
Figure 7. The amount of defecated faeces (g/day/rat) and volume of urine (ml/day/rat) by the control and NQX-treated rats during 14 days of the study.

Notes: Values are represented as mean ± SD. Comparison between control and NQX-treated rats was done using the ANOVA test. * p < 0.05; ** p < 0.01.

Table 2. Average values of leukocytes, erythrocytes and haemoglobin in control and NQX-injected animals

| Parameters             | Control       | Rats treated with NQX at 40 mg/kg |
|------------------------|---------------|-----------------------------------|
| Leukocytes (million /mm³) | 4.01 ± 0.3    | 4.06 ± 0.6                        |
| Erythrocytes (million/mm³) | 6.13 ± 0.4    | 6.23 ± 0.16                       |
| Haemoglobin (g/l)      | 130 ± 4.2     | 128 ± 6                           |

Note: Values are represented as mean ± SD.

Table 3. Biochemical parameters in control and NQX-treated animals at 40 mg/kg

| Parameters              | Control       | Rats treated with NQX at 40 mg/kg |
|-------------------------|---------------|-----------------------------------|
| Total Protein (g/l)     | 55 ± 0.7      | 54.2 ± 2.6                        |
| Glucose (mmol/l)        | 6.77 ± 0.7    | 6.98 ± 0.75                       |
| Creatinine (μmol/l)     | 47.2 ± 6.03   | 48.5 ± 7.8                        |
| Urea (mmol/l)           | 5.6 ± 0.3     | 5.7 ± 0.9                         |
| Bilirubin (μmol/l)      | 3.13 ± 0.55   | 3.7 ± 0.75                        |
| Total cholesterol (mmol/l) | 1.31 ± 0.2   | 1.45 ± 0.22                       |
| Triglycerides (mmol/l)  | 0.57 ± 0.19   | 0.58 ± 0.11                       |
| TGO (UI)                | 87.3 ± 10     | 89.7 ± 12                         |
| TGP (UI)                | 31.2 ± 8.5    | 30.6 ± 5                          |

Note: Values are represented as mean ± SD.
4. Discussion
In recent years, Quinoxaline and its derivatives have become amongst the molecules of interest due to their wide variety of biological activities and their therapeutic applications. To contribute to the identification of important therapeutic uses of these molecules, new quinoxalinic derivatives were synthesized and investigated for various pharmacological assays (Nakache et al., 2012, 2015). The objective of this study was to assess the toxicological profile of quinoxalinic derivative, NQX, in the Wistar rat.

To evaluate the compound safety for human’s trials, toxicological studies should be performed on animals first. In this work, the i.p. injection of NQX at different doses in Wistar rats for 14 days showed distinctive effects depending on the used dose.

In fact, we did found that the LD50 value for the NQX is 161.16 mg/kg. According to the International toxicity scale, NQX is ranked as moderate toxic substance (Claude, 1988; OCDE, 2000). In other work (Olayiwola et al., 2007), the LD50, which is the index of acute toxicity, was calculated for a series of some simple quinoxaline compounds and showed that the lowest LD50 was 74 mg/kg, i.p; whilst the highest LD50 was 160 mg/kg, i.p, with other compounds having LD50 values between the two doses. The LMD is 60 mg/kg since it is the minimal dose that causes the death of at least one treated rats. Toxicity symptoms that occur at this dose are characterized by several external clinical signs that increase in higher dose (from 60 to 300 mg/kg). Those symptoms include a decrease in body weight, food and water consumed and volume of urine and the amount of faeces.

In this experiment, the i.p. injection of 40 mg/kg of NQX in rats did not cause any animal death or clinical signs. Furthermore, injecting this dose did not cause any significant body weight changes in comparison to controls during all experiments (14 days). Our results also showed that the injection of the 40 mg/kg did not cause any significant changes in food and water consumption, or faecal and urinary excretion compared to control. Those results suggest that the NOAEL dose of NQX is 40 mg/kg.

To ensure the non-toxic effect of this dose, different haematological and biochemical analysis were performed at the end of the experiment (day 14). We showed that the injection of the NOAEL dose of NQX does not cause any significant differences in the number of leukocytes, erythrocytes and haemoglobin compared to control. Thus, the many physiological mechanisms controlled by these haematological parameters (respiration, immunology, etc.) remain normal.

Plasma Creatinine and urea levels are excellent markers of renal function. Any increase or decrease in their level can predict a possible renal dysfunction (Sirwal, Banday, Reshi, Bhat, & Wani, 2004). Our study demonstrates that creatinine and urea levels did not change in rats treated with NQX at the NOAEL dose, suggesting a normal renal function.

| Table 4. Average organ weights (g) in control and NQX (40 mg/kg)-treated rats |
|---------------------------------|-------------------------------|
| Organs             | Control | Rats treated with NQX at 40 mg/kg |
| Brain              | 1.55 ± 0.32 | 1.55 ± 0.36 |
| Kidney             | 1.6 ± 0.19 | 1.59 ± 0.12 |
| Spleen             | 1 ± 0.75 | 1.02 ± 0.7 |
| Pancreas           | 0.92 ± 0.12 | 0.9 ± 0.17 |
| Heart              | 1.16 ± 0.62 | 1.45 ± 0.6 |
| Liver              | 7.32 ± 1.95 | 7.36 ± 1.82 |

Values are represented as mean ± SD.
A transaminases enzyme has an elevated metabolic activity in cells. Any serum increase of these enzymes can reflect cell injury, especially in the liver (Kew, 2000). The average values of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic (TGP) remained within normal range even after NQX injection at the NOAEL dose. Liver function, assessed by the bilirubin level in serum, remained normal in the treated rats. Furthermore, the injection of the NOAEL dose of NQX did not cause any significant change in serum levels of total cholesterol, triglyceride, total protein and glucose compared to control, suggesting normal carbohydrate, lipid and protein metabolisms.

Moreover, our results show that NQX injection tested at a dose of 40 mg/kg did not change the weight of the different organs (liver, kidney, spleen, pancreas, heart and brain) compared to control.

In conclusion, the NQX LD50 is 161.16 mg/kg body weight in the Wistar rat. The i.p. administration of NQX did not cause any observable organ toxicity in rats and based on haematology and biochemistry serum results, the NQX NOAEL was fixed at 40 mg/kg in Wistar rat under the present experimental conditions.
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