*Advances in Biological Sciences Research, volume 22*
**7th International Conference on Biological Science (ICBS 2021)**

# LC50 and Effect of Sublethal Concentration of K2Cr2O7 on Different Developmental Stages of *Osteochilus vittatus*

Gratiana E. Wijayanti[1*] Sharon Hillary[1] Ani Septiani[1] Anisa Aulia[1] Anastasia E. Sintanora[1]

[1] *Faculty of Biology, Jenderal Soedirman University, Jl. Suparno No.63 Purwokerto Central Java 53122, Indonesia*
[*]*Corresponding author. Email: 1.bugrat@gmail.com*

**ABSTRACT**
Chromium in form of hexavalent had been reported to be genotoxic and carcinogenic, affects some physiological, and reproductive features. The ability of aquatic animals including fish to tolerate chromium may vary according to different developmental stages. Therefore, this research was conducted to (1) determine the $LC_{50}$ of $K_2Cr_2O_7$ for embryo, larvae, and juvenile of *Osteochilus vittatus*, and (2) evaluate the chronic exposure of sublethal concentration of $K_2Cr_2O_7$ in embryo and juvenile. The LC50 96h was determined using Probit analysis. Two experimental studies were conducted using a completely randomized design. In experiment-1: 5 groups of 2-cell stage embryos were exposed to, 0, 20, 40, 60, and 80 ppm of $K_2Cr_2O_7$, 8 units replicate each containing 200 embryos were provided, the parameters were hatching rate (HR) and survival rate (SR) of 4dph larvae. In experiment-2: three developmental stages i.e. 30dph, 60 dph, and 90 dph juveniles were exposed to 0 ppm, 2.5 ppm, and 5.00ppm of $K_2Cr_2O_7$, for 30 days; the observed parameters were total body length, body weight, and SR. The results showed that $LC_{50}$ for embryos was 100ppm, for larvae was 14.37ppm and for juveniles was 8.1ppm. The results of experiment-1 showed that the hatching rate of embryos treated with 0, 20, 40, 60, and 80 ppm of $K_2Cr_2O_7$ were 90.61±2.52%, 81.13±0.70%, 87,21±4.62%, 76.30±3.20%, and 77.30±4.12% respectively. The survival rate of 4 dph larvae of the treated embryos were 100%, 99.66±0.29%, 98.71±0.84%, 98.31±1.79, and 94.61±1.75 respectively, The results of experiment-2 showed that 30dph juvenile treated with 0ppm, 2.5ppm, and 5.00ppm of $K_2Cr_2O_7$ have a body length of 14.67±1.52mm, 12.33±5.51mm and 10.00±0.81 respectively; the treated 60dph juvenile have a body length of 26.75±3.88mm, 20.25±3.84mm, 20.62±3.85mm respectively; the treated 90dph juvenile have body length of 37.00±1.7mm, 35.00±2.16mm, and 33.33±1.52mm respectively. The body weights of juveniles exposed to $K_2Cr_2O_7$ were significantly lower ($p$<0.1) compare to the control group. The SR of the juveniles exposed to $K_2Cr_2O_7$ was significantly lower ($p$<0.01) compared to the control group. Based on all parameters it can be concluded that the $LC_{50}$ of $K_2Cr_2O_7$ in *O. vittatus* was varied according to its developmental stages and its effects was detrimental. Attention needs to be taken to regulating the release of effluent-containing chromium to the environment.

***Keywords:*** *Chromium, Embryo, Larvae, LC50, Osteochilus vittatus.*

## 1. INTRODUCTION

Chromium is one of the heavy metal presences in effluent originated from tanneries, mining, and stainless steel assembling and elastic assembling factory [1] Chromium is also present in batik waste water effluent [2]. Studies at two batik-producing Centres showed that water of Rowojembangan Creek, Kulon Progo contained total chromium of 0.18 – 0.303 ppm (0.123±0.090 ppm), and Cibentar River, Salem Brebes Regency contained total chromium of 0.013 – 0.219 ppm (0.084±0.060ppm) [2]. Chromium present in the

water enters the cells via the sulfate-anion channel [3] and can be accumulated in various tissue of fish. Chromium had been reported to be accumulated in the skin, gill, and muscle of *Oreochromis aureus* [4], *Hypophthalmichthys molitrix*, *Oreochromis nilotica*, *L. rohita*, *O. mossambica*, *Channa marulius* [5], and *Catla catla* [5, 6], *Labeo bata* and *Puntius sarana* [6].

Chromium in the form of hexavalent had been reported to be genotoxic and carcinogenic [7, 8], affects some physiological [9], and reproductive features [10]. Most studies on chromium toxicity were only conducted at a particular stage of development. In the aquatic environment, fishes are exposed to chromium throughout their life span. There is a lack of information on the effects of long-term exposure of chromium at the sublethal concentration on the development, health, and survival of aquatic animals. In addition, the ability of aquatic animals including fish to tolerate chromium may vary according to different developmental stages. Therefore, this research was conducted to (1) determine the $LC_{50}$ of $K_2Cr_2O_7$ for embryo, larvae, and juvenile of *Osteochilus vittatus*, and (2) evaluate the chronic exposure of sublethal concentration of $K_2Cr_2O_7$ in embryo and fry. *Osteochilus vittatus* was chosen as an experimental animal since this species is widely cultured for food needs. Thus, information from this research will be useful for the fish farmers as well as for decision-makers in designing policy to regulate the limit of chromium concentration allowed to be released to the environment.

## 2. METHODS

### *2.1. Animals*

The *O. vittatus* embryos and larvae were obtained from induced spawning at our laboratory. Pairs of brooders were induced to spawn using 0.5ml.kg$^{-1}$ body weight salmon GnRH analogue with domperidone (Syndel Laboratory, Vancouver Canada). As the spawning sign was observed, the brooders were removed from the aquaria then were gently stripped to obtain the oocytes and sperm. The oocytes and sperm were mixed to facilitate fertilization for 3 minutes, rinsed in slow running water then were incubated in the hatching aquaria. The juveniles age of 30 dph, 60 dph, and 90 dph were obtained from Pandak Freshwater fish breeding Centre in Banyumas Regency. The juveniles were acclimated for one week under laboratory conditions prior to the treatment. The juveniles were reared in 60x40x30cm aquaria filled with earth water and fully aerated for a continuous supply of Oxygen. The juveniles were fed with commercial fish pellets containing 41% protein, 5% lipid, 5% fiber, and 10% water. The food was given daily as much as 5% of total body mass. The water in the aquaria was siphoned and refreshed every 3 days. The fish were monitored daily at 7.00 – 8.00 and 16.00 – 17.00.

### *2.2. $LC_{50}$ Determination*

The tested chemical was $K_2Cr_2O_7$ (MW=294.21, Merk Cat.no. 024-002-00-6). The procedure for LC50 testing was conducted according to OECD [11, 12]. The 2-cells stage embryos were selected for the $LC_{50}$ test to make sure that only normal embryos were used in this study. A series of $K_2Cr_2O_7$ concentrations consisting of 0, 1, 5, 10, 20, 40, 80, 160, 340, 680, and 1260 ppm were prepared in triplicate. Two hundred embryos were placed into each replicate, the embryos were aerated. The number of hatched embryos was counted for mortality determination. The corrected embryo mortality (CM) was determined according to Equation (1).

$$CM = \frac{\%M \text{ of treated} - \%M \text{ of Control}}{100 - \%M \text{ of control}} \times 100\% \quad (1)$$

The LC50 of larvae was determined using 7 days post-hatching (dph) larvae. The tested concentration of $K_2Cr_2O_7$ were 0, 5, 10, 15, 20, 40, 60, and 80ppm. Three replicates each containing 10 larvae were provided for each test unit. The fries were not given food during the LC50 96h determination. The number of dead fries was recorded to determine the larvae mortality using the formulae as in Equation (1)

The juvenile LC50 was determined using fries with an average total body length of 9.91±0.14cm. The tested concentration of $K_2Cr_2O_7$ were 0, 5, 10, 15, 20, 40, 60, and 80ppm. The procedure was conducted for 4days, the number of dead juveniles was recorded every day for mortality determination according to Equation (1)

Data were subject to probit analysis according to Finney's methods [13]. The probit of mortality was denoted as *y* and the log of $K_2Cr_2O_7$ concentration was denoted as *x*. The *y* and *x* were the subjected to regression analysis to determine the $LC_{50}$. The probit analysis was performed using Microsoft Excel version 2016.

### *2.3. Effect of sub lethal exposure of $K_2Cr_2O_7$ on embryonic development*

Based on the embryonic $LC_{50}$, a completely randomized design experiment was conducted to examine the sublethal effect of $K_2Cr_2O_7$ on embryonic development of *O. vittatus* Five groups of 2-cells stage embryos were exposed to, 0, 20, 40, 60, and 80ppm of $K_2Cr_2O_7$, 8 units replicates each containing 200 embryos were provided, the measured parameters were hatching rate (HR) and survival rate (SR) of 4dph larvae. The HR and SR were determined using Equation (2) and Equation (3) respectively. The larvae were not fed since they still have a yolk.

$$HR = \frac{Number\ of\ hatched\ embryos}{Number\ of\ fertilized\ eggs} x100\% \quad (2)$$

$$SR = \frac{Number\ of\ 4dph\ larvae}{Number\ of\ hatched\ embryos} x100\% \quad (3)$$

### *2.4. Effect of sub lethal exposure of $K_2Cr_2O_7$ on Juvenile development*

Three developmental stages of juvenile i.e. 30dph, 60 dph, and 90 dph fry were exposed to 0, 2.5ppm, and 5.00ppm of $K_2Cr_2O_7$, for 30 days. The fries were reared in aerated aquaria and fed with commercial fish pellets as much as 3% of total biomass daily. The aquaria were siphoned every 3 days to maintain the water quality. Water temperature and pH were monitored every 7 days. The data in form of total body length, body weight, and SR were collected after 30 days of $K_2Cr_2O_7$ exposure.

### *2.5. Data Analysis*

The data in form of HR, SR, and total body length were tested for normality using the Kolmogorov-Smirnov test and homogeneity using Levene's test. The data were confirmed to be homogenous and normally distributed then were analyzed using Anova followed post hoc test (Tukey).

## 3. RESULTS

### *3.1. LC50 of Embryo, Larvae, and Juvenile*

The $LC_{50}$ for $K_2Cr_2O_7$ in *Osteochilus vittatus* was varied according to the developmental stages. The $LC_{50}$ for embryos was 100ppm, $LC_{50}$ for larvae was 14.37ppm (y=4.76x-0.507, $R^2$= 0.930) and $LC_{50}$ for juveniles was 8.1 ppm (y=8.853x-3.043, $R^2$= 0.847).

### *3.2. Effect of sub lethal exposure of $K_2Cr_2O_7$ on embryonic development*

The effect of $K_2Cr_2O_7$ exposure on embryonic development of *O. vittatus* was evaluated based on their HR and SR of 4dph larvae (yolk sac larvae). The HR of control embryos and those treated with 20, 40, 60, and 80ppm of $K_2Cr_2O_7$ were 90.61±2.52%, 81.13±0.70%, 87.21±4.62%, 76.30±3.20%, and 77.30±4.12% respectively. The HR of embryos exposed to 60ppm and 80ppm of $K_2Cr_2O_7$ were significantly lower than control embryos ($p<0.01$) and those exposed to 20ppm and 40pph of $K_2Cr_2O_7$ ($p<0.05$). The SR of 4 dph larvae of the control and the treated embryos were 100%, 99.66±0.29%, 98.71±0.84%, 98.31±1.79, and 94.61±1.75 respectively. The SR of 4 dph larvae was significantly lower than control and those exposed to 20 – 60ppm ($p<0.05$). These data showed that $K_2Cr_2O_7$ exposure affected embryonic development as indicated by the decrease of HR and SR. Morphological evaluation on prehatching embryos and newly hatched larvae showed that $K_2Cr_2O_7$ exposure induced embryos and larvae abnormality. Their body length was shorter with oedema at the pericardial cavity and abnormal vertebrae curvature showed by the bent tail and hook-like tail.

### *3.2. Effect of sub lethal exposure of $K_2Cr_2O_7$ on Juvenile development*

The exposure of 2.5ppm, and 5.0ppm K2Cr2O7 for 30 days to the 30dph, 60dph, and 90dph juvenile significantly decreased their SR and growth in concentration-dependent manner (Table 1). The lowest SR was observed in the juvenile exposed to 5.0ppm K2Cr2O7 regardless of their developmental stages (age). The growth of the survivor as measured by their total body length and body weight was also impaired.

**Table 1.** The Survival Rate of *O. vittatus* juveniles exposed to 0ppm (control), 2.5 ppm, and 5.00ppm $K_2Cr_2O_7$ for 30 days starting at 30dph, 60 dph, and 90dph

| Parameter | Concentration of $K_2Cr_2O_7$ | 60 dph | 90dph | 120dph |
|---|---|---|---|---|
| Survival Rate (%) | 0 (Control) | 88.83±11.39 | 83.33±5.77 | 40.83±6.31 |
| | 2.5 ppm | 68.33±3.33 | 79.17±6.87 | 7.50±8.77** |
| | 5.0 ppm | 18.33±14.78* | 39.17±9.95** | 5.00±4.30** |
| Total body length (mm) | 0 (Control) | 14.67±1.53 | 26.75±3.88 | 37.00±1.7 |
| | 2.5 ppm | 12.33±2.52 | 20.25±3.84 | 35.00±2.16 |
| | 5.0 ppm | 10.00±0.82 | 20.3±3.85 | 33.33±1.53 |
| Body weight (g) | 0 (Control) | 71.00±19.00 | 361.50±32,48 | 842.60±23.75 |
| | 2.5 ppm | 51.33±20.25* | 207.88±49.45** | 694.00±136.15 |
| | 5.0 ppm | 25.75±9.88* | 130.75±52.89** | 370.67±80.75** |

* $p<0.05$; ** $p<0.01$

## 4. DISCUSSION

The $LC_{50}$ value in the different developmental stages of *O. vittatus* is interesting. Initially, it was predicted that the $LC_{50}$ of the embryo will be lower than the larvae and juvenile because several studies suggested that the early stage of fish is more prone to toxicants [14]. The data in this study, however, did not match the prediction. A favourable explanation for such condition is that the embryos were exposed to $K_2Cr_2O_7$ at the 2-cells stage in which the developing embryos are protected by the chorion and the substance present in the perivitelline cavity. A study in *Oryzias latipes* showed that embryonated chorion (water hardened chorion) capable of accumulating cadmium exposed to the embryos and reduced the amount of this heavy metal reaching the embryo [15]. To test if the same condition also occurs in *O. vittatus*, in a separate experiment, we fertilized *O. vittatus* oocytes in media containing $K_2Cr_2O_7$ at a concentration up to 80ppm. The results showed that the fertilization rate and the HR of the embryos were comparable to the HR obtained from the sublethal exposure (data not shown).

The $LC_{50}$ of Larvae was lower than the embryo but higher than the juvenile. After hatching, the larvae are no longer protected by the chorion thus more exposed to $K_2Cr_2O_7$. On the first two days post-hatching the mouth of *O. vittatus* larvae is still closed and the movement is still limited. As the development proceeds, the postlarvae and juveniles become more active. An increase in swimming, breathing, and feeding activities increase the area of exposure to $K_2Cr_2O_7$ through the skin, gill lamella, and mouth epithelium. The wider surface area in contact with water containing $K_2Cr_2O_7$ will increase chromium intake and its accumulation in various tissue. This condition might in part contribute to lower $LD_{50}$ in the *O. vittatus* juvenile indicating that the juvenile of *O. vittatus* is less tolerant to $K_2Cr_2O_7$ compared to embryo and larvae. A similar condition was also reported in *Pagrus major* in which 1-day old fish were more tolerant to cadmium than older larvae [16].

The exposure of $K_2Cr_2O_7$ at a sublethal concentration significantly decreased the HR and the SR of 4dph larvae. $K_2Cr_2O_7$ dissolved in water will produce Cr(VI) which is capable to pass through plasma membrane using a concentration gradient of divalent anion in chloride phosphate anionic channel as a tetrahedral divalent $CrO_4^{2-}$ anion. This anion is structurally analogous to $PO_4^{3-}$, anions transported across cytoplasmic membranes through the chloride phosphate channel carrier, which integrates itself onto the organelle membrane structure and passes its content into various subcellular compartments [17]. Inside the cells, Cr(VI) can be reduced to intermediate forms such as Cr(V), Cr(IV), and Cr(III). In this reduction process, ROS are produced and lead to lipid peroxidation, DNA damage and apoptosis [18, 19, 20], disfunction of $Ca^{2+}$ mobility, cell cycle regulation, and metabolism [21]. These reactions might fail cell function related to developmental disturbance responsible for the decrease of HR. There is also evidence that $K_2Cr_2O_7$ exposure to embryos slows down embryonic development. In a normal laboratory condition (water temperature 26 – 28$^o$ C, pH 7 – 9, and saturated Oxygen), *O. vittatus* embryo hatch 23-25 hours post-fertilization [22]. In the current experiment, embryos exposed to 60ppm of $K_2Cr_2O_7$ hatched 35.2±0.0 hours post-fertilization, and those exposed to 80ppm of $K_2Cr_2O_7$ hatched 46.28±0.03 hours post-fertilization. The developmental disturbance may result in low quality of larvae and disruption of yolk utilization leads to reduced SR of the larvae.

The exposure of 5ppm $K_2Cr_2O_7$ to 30dph, 60dph, and 90dph *O. vittatus* juvenile for 30 days significantly decreased their survival. The survivors of this exposure have a significant decreased ($p<0.01$) in body weight (Table 1). The effect of chromium on fish growth has also been reported in *Platichthys stellatus*. In this species, exposure of 400ppb $K_2Cr_2O_7$ resulted in decrease in daily length gain and daily weight gain in 2 weeks [23]. A study in *Oreochromis niloticus* showed that exposure of 15µg/L- 3015µg/L for 28 days significantly reduced the survival rate [4]. $K_2Cr_2O_7$ exposed to fish are accumulated in the gill, skin, and muscle [4]. Accumulation of Chromium in the gill affect the basement membrane and submucosa, in the more severe condition the gill filaments undergo hypertrophy [20]. This condition caused the fish to avoid taking food and reduce oxygen intake. The low food and oxygen intake leads to growth inhibition and increase mortality.

Based on the result of this study, it can be concluded that the LC50 of $K_2Cr_2O_7$ for embryo, larvae, and juvenile of *O.vittatus* indicate that there is a differential tolerance to $K_2Cr_2O_7$ in this species. Exposure of sublethal $K_2Cr_2O_7$ disturbs embryonic development, slow down fish growth, and increased mortality.

## AUTHORS' CONTRIBUTIONS

GEW contributed on LC50 determination, data analysis and manuscript preparation, SH contributed on juvenile rearing and body length measurement, AS, AA and AES equally contributed on embryo incubation, HR and SR calculation.

## ACKNOWLEDGMENTS

This research was funded by Universitas Jenderal Soedirman BLU Research Scheme granted to GEW.

## REFERENCES

[1] S. DeFlora, Threshold mechanism and site specificity in chromium (VI) carcinogenesis,

Carcinogenesis, vol. 21, 2000, pp. 533-541. DOI: https://doi.org/10.1093/carcin/21.4.533

[2] G.E. Wijayanti, W. Lestari, Upaya pengembangan metode biodeteksi bahan pencemar limbah cair batik melalui kajian diversitas dan performa reproduksi ikan sungai, Research Report, Universitas Jenderal Soedirman, 2013.

[3] Y. Wang, H, Su, Y. Gu, X. Song, J. Zhao, Carcinogenicity of chromium and chemoprevention: a brief update, Onco Targets and Therapy, vol 10, 2017, pp. 4065 – 4079. DOI: https://doi.org/10,2147/OTT.S13922

[4] H.M. El-Shafei, Bioaccumulation of hexavalent chromium in tissue of a freshwater fish, Biochem Anal Biochem, vol. 5, 2016. DOI: 10.4172/2161-1009.1000272

[5] B. Kumar, D.P. Mukherjee, S. Kumar, Bioaccumulation of heavy metals in muscle tissue of fishes from selected aquaculture ponds in East Kolkata Wetlands, Ann Biol Res, vol. 2, 2011, pp. 125–134.

[6] T. Sanyal, A. Kaviraj, S. Saha, Toxicity and bioaccumulation of chromium in some freshwater fish, Human and Ecological Risk Assessment: An International Journal, vol. 23, 2017, pp. 1655-1667 DOI: 10.1080/10807039.2017.1336425

[7] F.C. Manning, L.J. Blankenship, J.P. Wise, J. Xu, L.C. Bridgewater, S.R. Patierno, Induction of internucleosomal DNA fragmentation by carcinogenic chromate: relationship to DNA damage, genotoxicity, and inhibition of macromolecular synthesis, Environ Health Perspect., vol. 102, 1994, pp. 159–167.

[8] A. Kortenkamp, M. Casadevall, S.P. Faux, A role for molecular oxygen in the formation of DNA damage during the reduction of the carcinogen chromium(VI) by glutathione, Arch Biochem Biophy, vol. 329, 1996, pp. 199–207.

[9] D.H. Ko, H.J Park, J.C Kang, Change of growth performance, hematological parameters, and plasma component by hexavalent chromium exposure in starry flounder, *Platichthys stellatus*, Fisheries and Aquatic Science, vol. 22, 2019. DOI: https://doi.org/10.1186/s41240-019-0124-5

[10] OEDC/OCDE, Fish Embryo Acute Toxicity (FET) Test. OEDC Guidelines for the testing of chemical. No. 236. 2013. OEDC. Paris

[11] OECD/OCDE, Fish acute toxicity testing, OEDC Guidelines for the testing of chemical, Section 2: Effect on Biotic System, Test Guideline No. 203, 2019 OECD, Paris.

[12] D.J. Finney, Probit Analysis, Cambridge, England, Cambridge University Press, 1952.

[13] J. Barbara, P. Sarnowski, M. Witeska, K. Ługowska, Disturbances of early development of fish caused by heavy metals (a review), Electronic Journal of Ichthyology, 2009, pp. 76-96.

[14] M.G. Doncel, M. Larrea, S.S. Fortún, D.E. Hinton, Influence of water hardening of the chorion on cadmium accumulation in medaka (Oryzias latipes) eggs, Chemosphere, vol. 52, 2003, pp.75-83.https://doi.org/10.1016/S00456535(03)00 227-3

[15] R. Kuroshima, S. Kimura, K. Date, Y. Yamamoto, Kinetic analysis of cadmium toxicity to red sea bream, *Pagrus major*, Ecotoxicology and Environmental Safety, vol. 25, 1993, pp. 300-314.

[16] A. Chiu, J.D. Robertson, J. Shi, W.K.P. Lee, R. Hill, T.P Wakeman, A. Katz, B. Xu, N.S. Dalal, C. Chen, N. Chiu, L. Donehower, Review of chromium (vi) apoptosis, cell-cycle-arrest, and carcinogenesis, J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, vol. 28, 2010, pp. 188–230. DOI: 10.1080/10590501.2010.504980

[17] D. Shi, B.H. Jiang, Antioxidant properties of apple juice and its protection against Cr(VI)-induced cellular injury, J Environ Pathol Toxicol Oncol, vol. 21, 2002, pp. 233–242.

[18] X.F, Wang, M.L. Xing, Y. Shen, X. Zhu, L.H. Xu. Oral administration of Cr(VI) induced oxidative stress, DNA damage and apoptotic cell death in mice, Toxicology, vol. 228, 2006, pp. 16–23.

[19] R. Zang, Y. Xiang, Q. Ran, X. Deng, Y. Xiao, L. Xiang, Z. Li, Involvement of calcium, reactive oxygen species, and ATP in hexavalent chromium-induced damage in red blood cells, Cell Physiol Biochem, 2014, vol. 34, pp. 1780-1791

[20] X.H. Zhang, X. Zhang, X.C. Wang, Chronic occupational exposure to hexavalent chromium causes DNA damage in electroplating workers, BMC Public Health, vol. 11, 2011.

[21] G.E. Wijayanti, P.S. Sugiarto, A. Nuryanto, Perkembangan embrio dan larva ikan nilem yang diinkubasi pada media dengan berbagai temperatur, Prosiding Semnas Basic Science, vol. 7, 2010, pp.180-187.

[22] D.H. Ko, H.J. Park, J.C. Kang, Change of growth performance, hematological parameters, and plasma component by hexavalent chromium exposure in starry flounder, *Platichthys stellatus*, Fisheries and Aquatic Science, vol. 22, 2019. DOI: https://doi.org/10.1186/s41240-019-0124-5

[23] K.M. Ashish, M. Banalata, Acute toxicity impact of hexavalent chromium on behavior and histopathology of gill, kidney and liver of the fresh water fish *Channa puntatus*, Environ Toxicol Pharmacol, vol.26, 2008, pp, 136-141. DOI: 10.1016/j.etap.2008.02.010