PERGAMON

Toxicology in Vitro 17 (2003) 525–532

Toxicology in Vitro

www.elsevier.com/locate/toxinvit

Ecotoxicological evaluation of carbamazepine using six different model systems with eighteen endpoints

A. Jos[a,b], G. Repetto[a,b,*], J.C. Rios[c], M.J. Hazen[d], M.L. Molero[d], A. del Peso[a], M. Salguero[a], P. Fernández-Freire[d], J.M. Pérez-Martín[d], A. Cameán[b]

[a]*National Institute of Toxicology, PO Box 863, 41080—Sevilla, Spain*
[b]*Area of Toxicology, Universidad de Sevilla, Spain*
[c]*CITUC, School of Medicine, Catholic University of Chile, Chile*
[d]*Department of Biology, Universidad Autónoma de Madrid, Spain*

Accepted 1 June 2003

Abstract

The occurrence of pharmaceutically active compounds in the aquatic environment has been recognized as one of the emerging issues in environmental chemistry. However, the ecotoxicological effects of pharmaceuticals have still not been researched adequately. Carbamazepine, an anticonvulsant commonly present in surface and groundwater, was studied, using six ecotoxicological model systems with eighteen endpoints evaluated at different exposure time periods. The battery included the immobilization of *Daphnia magna,* bioluminescence inhibition in the bacterium *Vibrio fischeri*, growth inhibition of the alga *Chlorella vulgaris*, and micronuclei induction and root growth inhibition in the plant *Allium cepa*. Cell morphology, neutral red uptake, total protein content, MTS metabolization, lactate dehydrogenase leakage and activity and glucose-6-phosphate dehydrogenase activity were studied in the salmonid fish cell line RTG-2. The total protein content, LDH activity, neutral red uptake and MTT metabolization in Vero monkey kidney cells were also investigated. The most sensitive system to carbamazepine was the Vero cell line, followed by *Chlorella vulgaris*, *Vibrio fischeri*, *Daphnia magna*, *Allium cepa*, and RTG-2 cells. EC_{50} values from 19 μM in Vero cells at 72 h to more than 1200 μM in other systems, were obtained. Comparing the concentrations in water and the toxicity quantified in our assay systems, carbamazepine is not expected to produce acute toxic effects in the aquatic biota under these circumstances, but chronic and synergistic effects with other chemicals cannot be excluded.

Keywords: Alternatives; Battery; Ecotoxicology; Carbamazepine; Cytotoxicity; Contamination

1. Introduction

The occurrence and fate of pharmaceutically active compounds in the aquatic environment has been recognized as one of the emerging issues in environmental chemistry (Heberer, 2002). Pharmaceuticals are employed in human and veterinary medicine, agriculture and aquaculture. They are often resistant to biodegradation since metabolic stability is necessary for pharmacological action. Certain pharmaceuticals are also highly water soluble, and removal during wastewater treatment will be limited for such compounds. Aquatic biota are consequently exposed to more than 80 pharmaceuticals and drug metabolites as they enter the aquatic environment (Dietrich et al., 2002).

Although pharmaceuticals are specifically designed in terms of crop, farm animal or human target structures, they can also be active in non-target organisms where they elicit non-expected effects. Even slight, non-significant influences on single components within regulatory cascades, which would not result in any acutely discernible effect, might ultimately affect a whole

Abbreviations: EC_{50}, mean effective concentration; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium,inner salt; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

* Corresponding author. Tel.: +34-954-371233; fax: +34-954-370262.

E-mail address: repetto@us.es (G. Repetto).

0887-2333/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0887-2333(03)00119-X

population by their negative consequences on fitness: disturbances in hormonal homeostasis (endocrine disruption), immunological status, signal transduction or gene activation, for example (Seiler, 2002).

According to the basal cytotoxicity concept, a majority of chemicals cause toxicity by means of basal cytotoxicity, while a clear minority cause toxicity by interference with either organ-specific cell functions or extracellular bodily functions. According to this reductionistic view, the toxicity of a compound can be broken down into a number of elements, each of which can be identified and quantified in appropriate model systems (Ekwall, 1994). This makes pharmaceuticals a group of environmental pollutants to be taken into account in environmental risk assessment procedures.

The lack of ecological data on pharmaceuticals in general and antiepileptics in particular has been shown by several authors (Jones et al., 2002; Halling-Sorensen et al., 1998). Carbamazepine is an antiepileptic drug mainly metabolised into the active compound carbamazepine-10,11-epoxide and further into other inactive compounds, principally glucuronides. Aproximately 3% is excreted unchanged in urine, and is not significantly removed (less than 10%) during sewage treatment, resulting in the contamination of the receiving waters (Ternes, 1998; Andreozzi et al., 2002; Heberer, 2002; Jones et al., 2002).

The antiepileptics carbamazepine and primidone represented the most dominant of all investigated drugs in well treated domestic effluents in Arizona and California. Removal of the drugs did not seem to occur during travel times of more than 6 years in the subsurface (Drewes et al., 2002). Carbamazepine has been detected in municipal sewage treatment plant effluents (6.3 μg/l; Ternes, 1998), surface water (up to 1075 ng/l), groundwater (up to 1.1 μg/l) and drinking water (30 ng/l) (Heberer, 2002).

In order to study the effects of carbamazepine in the environment, a test battery has been applied. Such a battery should represent a wide range of organisms belonging to different trophic levels. Six ecotoxicological model systems with eighteen endpoints were evaluated at different exposure time periods. The systems included the immobilization of the cladoceran *Daphnia magna* (1st consumer), bioluminescence inhibition in the marine bacterium *Vibrio fischeri* (decomposer), growth inhibition of the alga *Chlorella vulgaris* (producer), micronuclei induction and mitotic and growth inhibition in the plant *Allium cepa* (producer). Total protein content, neutral red uptake, lactate dehydrogenase (LDH) activity and MTT metabolization were investigated in Vero monkey kidney cells (model of 2nd consumer). Neutral red uptake, total protein content, MTS metabolization, LDH leakage and activity, apoptosis induction and changes in morphology were studied in the RTG-2 cell line (model of 1st consumer), derived from of rainbow trout gonad (*Oncorhynchus mykiss*).

2. Materials and methods

2.1. Toxicant exposure

Stock solutions of carbamazepine (Sigma) were prepared in dimethylsulfoxide, the concentration of the solvent in medium being less than 1%, including the control groups. The exposure solutions were prepared before use in the different culture media according to the appropriate assay, and sterilized by filtration through a 0.22 μM Millipore® filter. After replacing the medium with the exposure solutions the systems were incubated for the adequate time period.

2.2. Model systems

Growth inhibition of the algae *Chlorella vulgaris var viridis* was evaluated in 96-well culture plates seeded with 200 μl/well of a 1,000,000 cells/ml algae culture in exponential growth phase, using constant agitation and a temperature of 22 °C, under a water saturated sterile atmosphere containing 5% CO_2 and a cold light source of 8000 lux. Absorbency at 450 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). As a quality criteria the control cultures must grow at least 10 times in 48 h (Ramos et al., 1996).

Bulbs of the onion *Allium cepa* L (15–30 g) were grown in the dark at a constant temperature of 25±0.5 °C. The bases of the bulbs remained submerged in filtered tap water (renewed every 24 h), aereated by continuous bubbling at a rate of 10–20 ml/min. (González-Fernández et al., 1971). The experiments started when meristem roots reached 15–20 mm. Five bulbs and three roots per bulb were used for each treatment. Root meristems of *A. cepa* were exposed for 48 h and then fixed in a mixture of ethanol/acetic acid (3/1 vol/vol) at 4 °C overnight. Finally, they were stained with acetic orcein (2%) according the method of Tjio and Levan (1950) and crushed in 50% acetic acid for the analysis of mitotic index and micronuclei, (Jos et al., in press). The mitotic index was calculated by counting the number of cells in mitosis within a field in relation to the total number of cells. The micronuclei frequencies were calculated from the number of cells where a separate fragment of the nuclei was observed. The assay was completed by measuring the length of the root bundles after 72 h of carbamazepine treatment. The control sets grew 8.8 times in 3 days.

Daphnia magna clone A (gift from Dr. Muñoz-Reoyo, CISA, Spain) was maintained at 20 °C and fed with *Chlorella vulgaris*. Acute toxicity immobilization tests were performed in standard reference water according

to OECD Guideline 202 (1993) in replicate groups of 10 neonates in 25 ml, contained in 70 ml polystyrene flasks (Costar, Cambridge, MA, USA).

Bioluminescence inhibition in the marine bacterium *Vibrio fischeri* was evaluated according to Cordina et al. (1993) by using freeze-dried bacteria (Microbics Corp. Carlsbad, USA) incubated at 15 °C.

Vero monkey kidney cells were grown at 37 °C in 75 cm^2 flasks (Costar, Cambridge, Ma, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Biochrom, Berlin, Germany). Cells were seeded at a density of 14,000 cells/ml into 24-well culture plates and incubated for 24 h. After removing the culture medium and washing in phosphate buffered saline, the cell cultures were exposed to 1 ml/well of increasing doses of carbamazepine. Cell number (to evaluate cell proliferation and/or detachment) was quantified by measuring the total cellular protein content (TPC), using bovine serum albumine (Sigma) as standard, by the method of Bradford (1976). Neutral red uptake (NRU) was measured according to Borenfreund and Puerner (1984) and intracellular LDH (EC1.1.1.27) activity as described by Vassault (1983). Cell viability was measured by the MTT metabolization assay according to a procedure based on Carmichael et al. (1987). MTT (5 mg/ml) was dissolved in PBS, sterilized by filtration through a 0.22 μM Millipore® filter and stored at 4 °C. After the different treatments, cells were washed twice with PBS, treated with 1.5 ml MTT (0.5 mg/ml in DMEM) per well and incubated for 2 h at 37 °C to allow MTT metabolization. The formazan produced was dissolved with acidic isopropanol. All the determinations were carried out in the same culture plates as used for exposure. Absorbencies were measured on a Spectrafluor microplate reader (Tecan, Austria).

RTG-2 cells, derived from rainbow trout gonad (*Oncorhynchus mykiss*), (gift from Dr Castaño, CISA, Spain) were grown in Eagle's Medium supplemented with 10% foetal calf serum (Biochrom). RTG-2 cells in exponential growth phase were plated at a density of 8000 cells/well in 96-well tissue-culture plates (Costar). After 24 h at 20 °C, the cultures received 0.2 ml medium containing the test chemical and were further incubated for 24, 48 or 72 h (Castaño et al., 2000). Cell number was quantified in situ, according to total cellular protein content (TPC), using Coomassie brilliant blue G-250 (Repetto and Sanz, 1993) in the same 96-well tissue-culture plates in which exposure originally took place (Repetto et al., 1993). Absorbency at 620 nm was read on a Multiscan RC plate reader (Labsystem, Helsinki, Finland). Neutral red uptake (NRU) was evaluated according to Babich and Borenfreund (1987). The MTS tetrazolium reduction assay was performed according to a procedure based on Baltrop et al. (1991). The MTS tetrazolium compound is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium. LDH (EC 1.1.1.27) activity in cells and in culture medium was determined according to Duffy and Flint (1987) and G6PDH activity was determined as described by Kornberg and Horecker (1955) and García Alfonso et al. (1998). For the morphological study, RTG-2 cells were seeded in Lab-Tek® tissue culture chamber slides (Nunc, Inc., Naperville, IL). They were then exposed to carbamazepine for 24, 48 and 72 h, fixed in 70% methanol and stained with Mayeŕs hematoxylin and eosin or subjected to in situ hybridization (TUNEL) to detect induction of apoptosis (Enzo, Diagnostics, Farmingdale, US).

2.3. Calculations and statistical analysis

All experiments were performed at least three times and at least in duplicate per concentration. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. EC_{50} values were determined by probit analysis.

3. Results and discussion

The effects of carbamazepine were investigated using six ecotoxicological model systems with eighteen endpoints. The alterations in a variety of systems including vegetables, bacteria, a crustacean, and cell cultures from monkey and fish origin were evaluated at different exposure time periods. The proliferation of the freshwater algae *Chlorella vulgaris* in 96-well microtiter plates *was* inhibited in a concentration-dependent way, showing EC_{50} values of 470 μM at 24 h and of 155 μM at 48 h (Fig. 1a). In contrast to our results, carbamazepine has been reported neither to produce growth inhibition nor to accumulate in *Ankistrodemus braunii* and *Selenastrum capricornutum* cultures (Andreozzi et al., 2002).

A similar range of effects was observed for root growth and mitotic index inhibition in meristematic cells of *Allium cepa*, with EC_{50} of 447 μM and 498 μM, respectively (Fig. 1b). This reduction was only statistically significant for high concentrations of carbamazepine (>100 μM). Similar results were previously reported in cultured mammalian cells since concentrations of carbamazepine above the therapeutic range inhibited the proliferation of murine lymphoid cells (Title and Schaumann, 1992) and decreased the mitotic and proliferation indices in human lymphocyte cultures (Awara et al., 1998). The mechanisms responsible for the cytostatic activity of carbamazepine are not yet fully studied, but could in part be attributed to the increase in the length of interphase period induced by this compound (Dulout and Olivero, 1984).

The genotoxic potential of carbamazepine has been poorly investigated and is not well understood. Our data did not reveal any statistically significant difference in the frequencies of micronuclei and aberrant anaphase-telophase cells in bulbs of *Allium cepa* (data not shown), even when high concentrations were tested. This finding is in agreement with the results of Sinues et al. (1995), who failed to detect any significant increase in cytogenetic parameters such as chromosome aberrations and micronuclei in humans. However, this does not support the findings of other investigators who reported a significantly higher frequency of chromosome aberrations (Herha and Obe, 1976) and sister chromatid exchanges (Awara et al., 1998) in patients receiving carbamazepine.

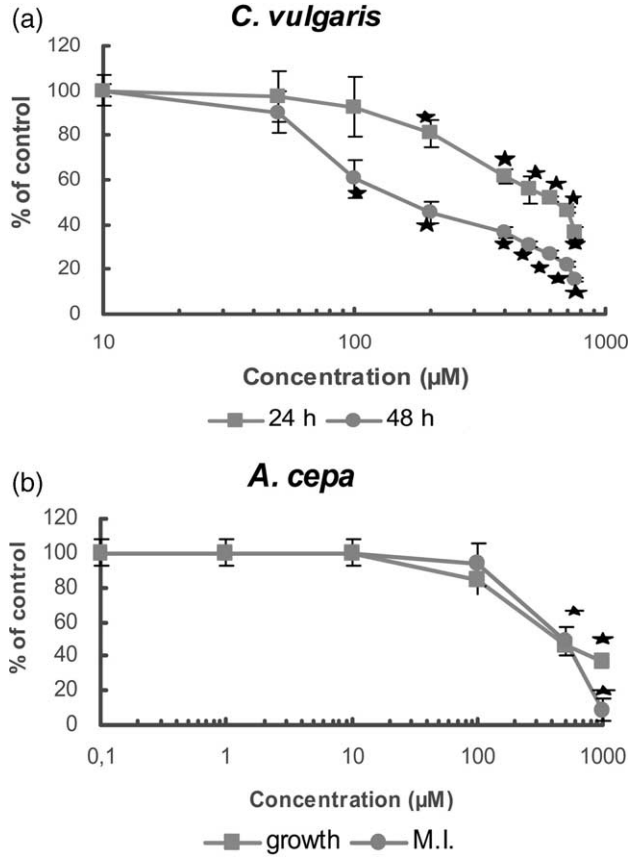

Fig. 1. (a) *Chlorella vulgaris* proliferation after exposure to different concentrations of carbamazepine for 24 (■) and 48 h (•) and (b) *Allium cepa* growth (γ) and mitotic index (•) after exposure to different concentrations of carbamazepine for 72 and 48 h respectively. Data expressed in % of the unexposed controls. * Indicates significant difference from control value ($P<0.01$).

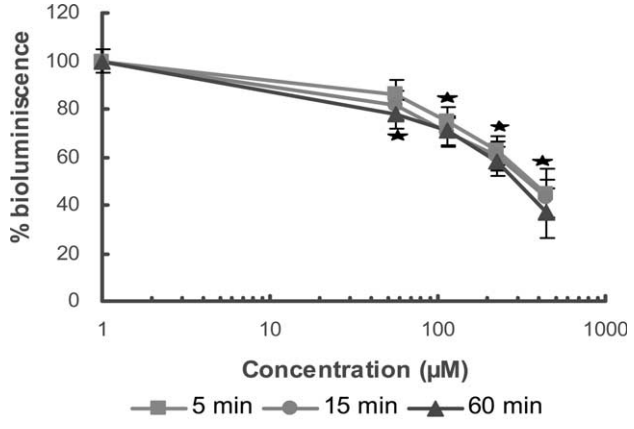

Fig. 2. Bioluminescence inhibition of *Vibrio fischeri* bacteria after exposure to different concentrations of carbamazepine for (a) 5 min (■), (b) 15 min (•) or 60 min (▲). Data expressed in% of the unexposed controls. * Indicates significant difference from control value ($P<0.01$).

Bioluminescence in *Vibrio fischeri* bacteria (Fig. 2) was inhibited with EC_{50} values of 370, 332 and 271 µM after 5 min, 15 min and 60 min, respectively. The results of this study also show an intermediate sensitivity of the crustacean *Daphnia magna* with EC_{50} values of 475 µM at 24 h and of 414 µM at 48 h of exposure (Fig. 3).

The Vero monkey cell line was the most sensitive model in this study, probably because carbamazepine was designed for a mammalian (human) target. Among the biomarkers analysed, the uptake of neutral red was the most sensitive at 72 h, followed by MTT metabolization (Fig. 4). Cell proliferation and intracellular LDH activity were less sensitive but in a similar time-dependent pattern. Cell proliferation was inhibited with EC_{50} values between 34 and 76 µM, neutral red uptake between 19 and more than 100 µM, MTT metabolization in a similar range and LDH activity between 39 and 72 µM (Table 1).

The results obtained for the different endpoints evaluated on RTG-2 cells are presented in Fig. 5. With EC_{50} values between 473 and 496 µM, the neutral red uptake was the most sensitive indicator, followed by MTS metabolization, total protein content and G6PDH activity, both with a similar sensitivity.

LDH leakage, a universally accepted marker of membrane permeability and cell death, is not increased to more than 8% at the highest concentration used, confirming the stabilization of the cytoplasmic

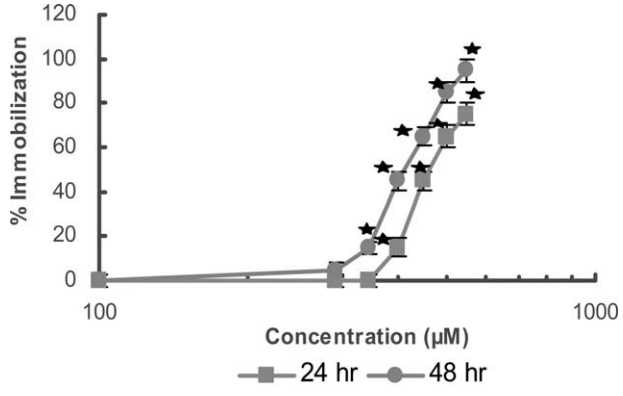

Fig. 3. *Daphnia magna* immobilization after exposure to different concentrations of carbamazepine for (a) 24 h (■) and (b) 48 h (•). Data expressed in % of the unexposed controls. * Indicates significant difference from control value ($P<0.01$).

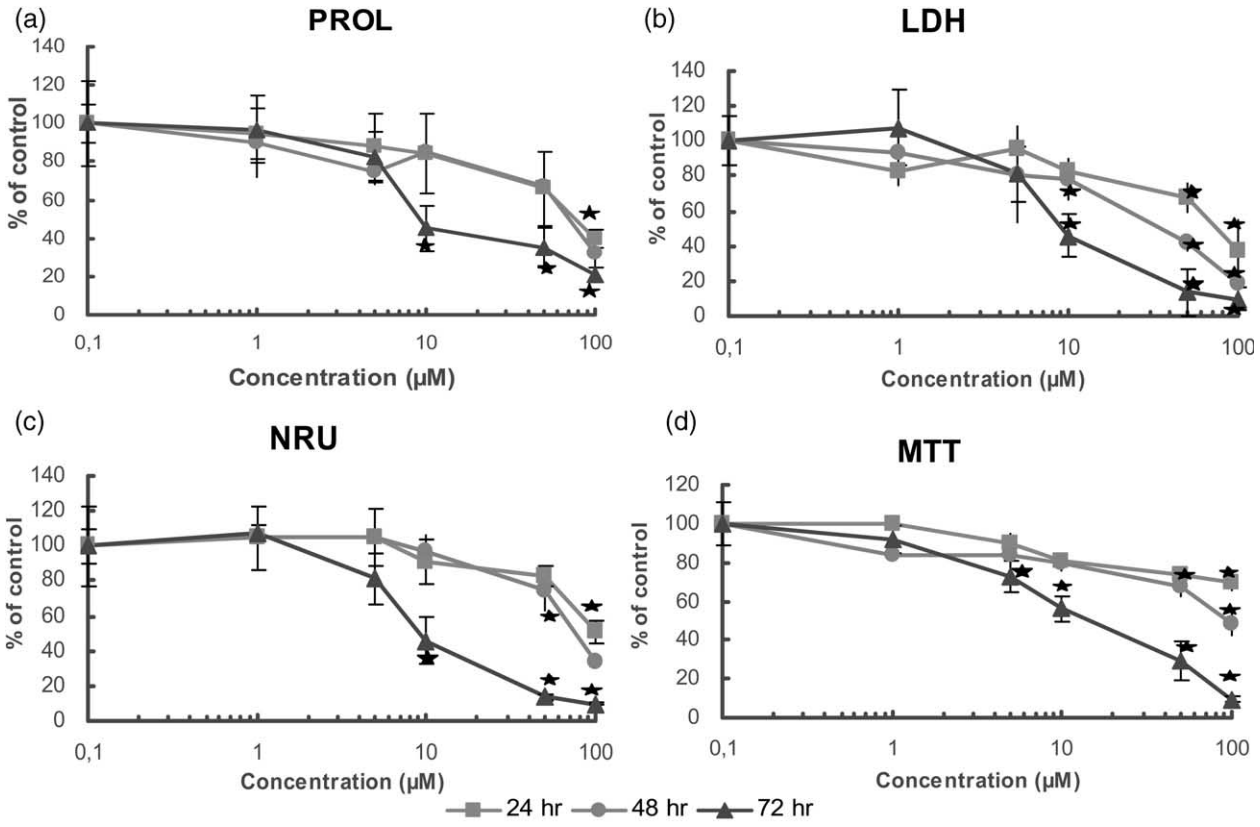

Fig. 4. Vero monkey cell culture (a) protein content, (b) LDH activity, (c) neutral red uptake and (d) MTT metabolization after exposure to different concentrations of carbamazepine for 24 h (■), 48 h (●) or 72 h (▲). Data expressed in % of the unexposed controls. * Indicates significant difference from control value ($P < 0.01$).

Table 1
Toxic effects of carbamazepine on the different models and biomarkers of the proposed ecotoxicological battery

Model system	Origin	Indicator	24 h	48 h	72 h
Chlorella vulgaris	Unicel. Algae	Growth	469.5	155	–
Allium cepa	Terrestrial Plant	Root growth	–	–	447
		Mitotic Index	–	498	–
		Micronuclei Induction	Not detected	–	–
Vibrio fischeri	Bacteria	Bioluminescence	370[a]	332[a]	272[a]
Daphnia magna	Cladoceran	Immobilization	475	414	–
Vero cell line	Monkey	Total protein content	76.2	62.9	33.6
		Neutral red uptake	>100	81.4	19
		MTT metabolization	>100	94.2	24.6
		LDH activity	72	67	38.5
RTG-2 cell line	Rainbow Trout	LDH leakage	>1200	–	–
		LDH activity	>1200	–	–
		Total protein content	832	713	933
		Neutral red uptake	496	472.6	495
		MTS metabolization	698	608.6	601.4
		G6PDH Activity	853	716.7	687

EC_{50} (μM) values are given.
[a] Values referred to 5, 15 and 60 min exposure times respectively.

membrane and good viability of the cultures within the range of exposure concentrations. EC_{50} values of up to 1200 μM for LDH leakage and intracellular activity were obtained. The stabilization of the cytoplasmic membrane to lactate dehydrogenase leakage has been previously described for lithium (Repetto et al., 2001), another psychotropic drug. However, lithium stimulated lysosomal function, but carbamazepine was a clear inhibitor. Carbamazepine blocks the voltage-dependent Na^+, delayed K^+, and transient Ca^{2+} currents and

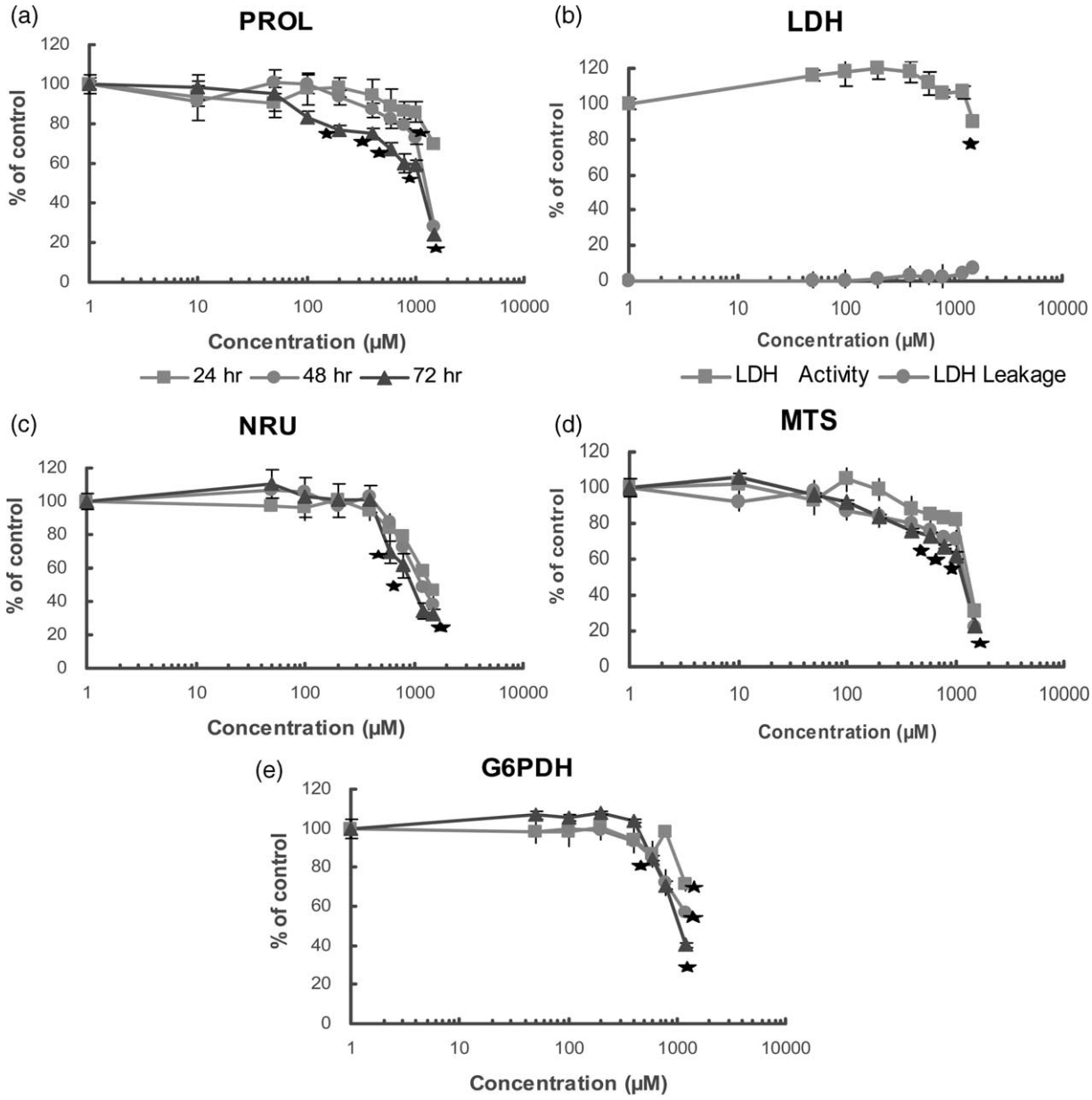

Fig. 5. Effects of carbamazepine in RTG-2 cells on cell culture (a) protein content, (b) LDH leakage and activity, (c) neutral red uptake, (d) MTS metabolization and (e) G6PDH activity after exposure to different concentrations of carbamazepine for 24 h (■), 48 h (•) or 72 h (▲). Data expressed in % of the unexposed controls. * Indicates significant difference from control value ($P < 0.01$).

quantal transmitter release, but does not show lysosomotropism (Matsumoto et al., 1998).

Morphological changes induced by carbamazepine were also investigated in RTG-2 cells (Fig. 6). The control cultures show fusiform cells, arranged in plaques and disposed in parallel. They have well defined borders, eosinophilic cytoplasm and central nuclei. The morphological alterations were evident only at high carbamazepine concentrations, particularly from 600 μM. The changes included loss of cells, induction of cellular pleomorphism, and hydropic degeneration of the cytoplasm (cellular swelling). When cells were very damaged, a prominent feature in the morphological study was the visualization of nucleolar structures, possibly due to chromatine condensation around these organelles. The induction of apoptosis confirmed by in situ hybridization (TUNEL) was evident after 24 h exposure with concentrations of carbamazepine of up to 600 μM.

One important piece of information needed in risk assessment is the concentration range at which a chemical produces adverse effects on organisms. Without this information we can neither make predictions nor establish safety factors. According to the basal cytotoxicity concept, a majority of chemicals cause toxicity by means of basal cytotoxicity, while a clear minority

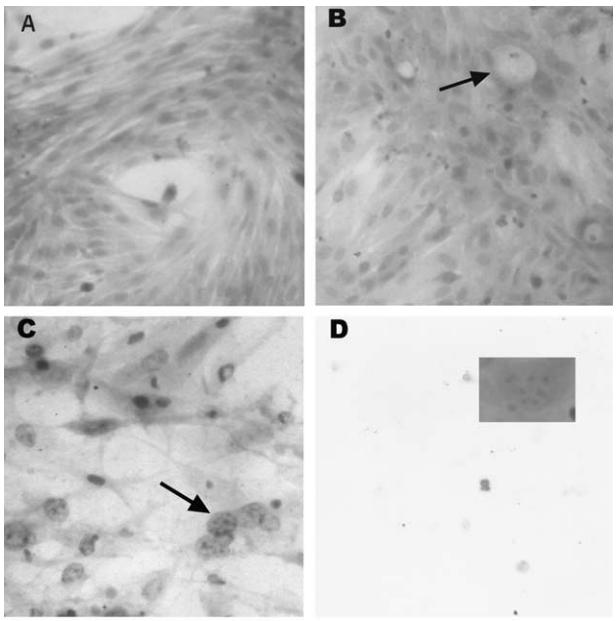

Fig. 6. Morphology of a (A) control culture of RTG-2 cells, showing fusiform cells, with eosinophilic cytoplasm and central nucleus, arranged in plaques and disposed in parallel. (B) Culture treated with 600 μM carbamazepine. Changes included loss of cells, induction of cellular pleomorphism, and hydropic degeneration (→) of the cytoplasm (cellular swelling). (C) A prominent feature in the morphological study was the visualization of nucleolar structures (→), due to chromatine condensation around these organelles. (D) The induction of apoptosis confirmed by *in situ* hybridization (TUNEL) was evident after 24 h exposure with concentrations of carbamazepine of up to 600 μM. Insert showing nucleolar structures.

cause toxicity by interference with either organ-specific cell functions or extracellular bodily functions (Ekwall, 1994). According to this reductionistic view, it seems possible that a limited number of cell lines or isolated cells from invertebrates and vertebrates may be sufficient for basal cytotoxicity screening. In the case of aquatic organisms, extrapolation can be performed easily, since concentrations in water can be more directly compared with concentrations in culture medium. For this reason, fish cell lines in particular are becoming more frequently employed as surrogates of whole-animal ecotoxicity testing with relevant (sub)-lethal endpoints (Castaño et al., 2003).

There are very few reports available on the effects of carbamazepine on aquatic organisms. This compound is considered toxic (EC_{50} 1–10 mg/l) for cnidaria and non-toxic ($EC_{50} > 100$ mg/l) for crustacea and fish (Jones et al., 2002), which is in accordance with the results of this study. Comparing the EC_{50s} (Table 1) at 48 h, the most sensitive system was the Vero cell line, followed by *Chlorella vulgaris*, *Vibrio fischeri*, *Daphnia magna*, *Allium cepa*, and RTG-2 cells. EC_{50} values were obtained from 19 μM in Vero cells at 72 h, to more than 1200 μM in other systems. Other chemicals have been found to produce a very different profile of effects on the diverse models (Repetto et al., 2001). Regarding the present European legislation on the classification and labeling of chemicals (92/32/EEC), carbamazepine should be classified, according to our results, as "R52/53 Harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment".

The complexity of the obtained results, with very different effects according to the test system and exposure period employed, shows that a single bioassay will never provide a full picture of the quality of the environment. A battery of test systems and indicators would be representative of a wide range of organisms. For the time being, a minimum ecotoxicological test battery should at least include bacteria, vegetables, invertebrates and mammalian and not-mammalian cells (Repetto et al., 2001).

According to the concentration of carbamazepine found in water samples, a calculated ratio between the predicted environmental concentration (PEC) and the predicted no-effect environmental concentration (PNEC), which is smaller than one for this compound (Jones et al., 2002), and the results obtained with the proposed test battery, carbamazepine is not expected to produce acute toxic effects on the aquatic biota under these circumstances. However, chronic effects and synergistic effects with other chemicals cannot be excluded and should be further investigated.

Acknowledgements

The support of the Spanish Ministry of Science and Technology, project AMB99-0279, is gratefully acknowledged. A. Jos is the recipient of a grant for the formation of research personal from the Ministry of Science and Technology. The authors thank S Jimenez for technical assistance.

References

Andreozzi, R., Marotta, R., Pinto, G., Pollio, A., 2002. Carbamazepine in water: persistence in the environment, ozonation treatment and preliminary assessment on algal toxicity. Water Research 36, 2869–2877.

Awara, W.M., El-Gohary, M., El-Nabi, S.H., Fadel, W.A., 1998. In vivo and in vitro evaluation of the mutagenic potential of carbamazepine: does melatonin have anti-mutagenic activity? Toxicology 125, 45–52.

Babich, H., Borenfreund, E., 1987. In vitro cytotoxicity of organic pollutants to bluegill sunfish (BF-2) cells. Environmental Research 42, 229–237.

Baltrop, J.A., Owen, T.C., Cory, A.H., Cory, J.G., 1991. 5-((3-Carboxyphenyl)-3-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 2-(4,5-dimethylthiazolyl)-2,5-diphenylterazolium bromide (MTT) reducing to purple water soluble formazan as cell-viability indicators. Bioorganic and Medicinal Chemistry Letters 1, 611.

Borenfeund, E., Puerner, J.A., 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR90). J. Tissue Cult. Methods 9, 7–9.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Carmichael, J., DeGraff, W.G., Gazdar, A.F., Minna, J.D., Mitchell, J.B., 1987. Evaluation of a tetrazolium-based semiautomated colorimetric assay; assessment of chemosensitivity testing. Cancer Research 47, 936–942.

Castaño, A., Sanchez, P., Llorente, M.T., de la Carballo, M., Torre, A., Muñoz, M.J., 2000. The use of alternative systems for the ecotoxicological screening of complex mixtures on fish populations. The Science of The Total Environment 247, 337–348.

Castaño, A., Bols, N., Braunbeck, T., Dierickx, P., Halder, M., Isomaa, B., Kawahara, K., Lee, L., Mothersill, C., Pärt, P., Repetto, G., Riego, J., Rufli, H., Smith, R., Word, C., Segner, H., 2003. The use of fish cells in ecotoxicology. The Report and Recommendations of ECVAM Workshop 47. Alternatives to Laboratory Animals 31, 317–351.

Cordina, J.C., Pérez-Garcia, P., Romero, P., Vincente, A., 1993. A comparison of microbial bioassays for the detection of metal toxicity. Archives of Environmental Contamination and Toxicology 25, 250.

Dietrich, D.R., Webb, S.F., Petry, T., 2002. Hot spot pollutants: pharmaceuticals in the environment. Toxicology Letters 131, 1–3.

Drewes, J.E., Heberer, T., Reddersen, K., 2002. Fate of pharmaceuticals during indirect potable reuse. Water Sci. Technology 46, 73–80.

Duffy, P.A., Flint, O.P., 1987. In vitro dermal irritancy tests. In: Atterwill, C.K., Steele, C.E. (Eds.), In Vitro Methods in Toxicology. Cambridge University Press, Cambridge, pp. 279–297.

Dulout, F.N., Olivero, O.A., 1984. Anaphase, telophase analysis of chromosomal damage induced chemicals. Environmental Mutagenesis 6, 216–218.

Ekwall, B., 1994. The basal cytotoxicity concept. In: Goldberg, A.M., van Zupthen, L.F.M. (Eds.), Alternative Methods in Toxicology and the Life Sciences, Vol 11—The World Congress on Alternatives and Animal Use in the Life Sciences: Education, Research, Testing. Mary Ann Liebert Inc. Publishers, New York, pp. 721–725.

García-Alfonso, C., Repetto, G., Sanz, P., Repetto, M., López-Barea, J., 1998. Direct determination of glutathione S-transferase and glucose-6-phosphate dehydrogenase: activities in cells cultured in microtitre plates as biomarkers for oxidative stress. Alternatives To Laboratory Animals 26, 321–330.

González-Fernández, A., Giménez-Martín, G., de la Torre, C., 1971. The duration of the interphase periods at different temperatures in root tip cells. Cytobiologie 3, 367–371.

Halling-Sorensen, B., Nors-Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten-Lutzhoft, H.C., Jorgensen, S.E., 1998. Ocurrence, fate and effectos of pharmaceutical substances in the environment—A Review. Chemosphere 36, 357–393.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters 131, 5–17.

Herha, J., Obe, G., 1976. Chromosomal damage in epileptics on monotherapy with carbamazepine and diphenylhydantoin. Human Genetics 34, 255–263.

Jones, O.A.H., Volvoulis, N., Lester, J.N., 2002. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. Water Research 36, 5013–5022.

Jos, A., Repetto G., Hazen, M.J., Ríos, J.C., Molero, M.L., del Peso A., Salguero, M., Fernández-Freire, P., Pérez-Martín, J.M., Repetto, M. In vitro evaluation of pentachlorophenol using Allium cepa, SH-SY5Y human neuroblastoma and RTG-2 salmonid fish cell lines. Aquatic Ecosystem Health and Management (in press).

Kornberg, A., Horecker, B.L., 1955. Glucose-6-phosphate dehydrogenase: enzymes of carbohydrate metabolism. Methods in Enzymology 1, 323–327.

Matsumoto, Y., Enomoto, K., Moritake, K., Maeno, T., 1998. Effects of carbamazepine on nerve activity and transmitter release in neuroblastoma-glioma hybrid cells and the frog muscular junction. Cell Biol. Toxicol. 14, 191–198.

OECD, 1993. OECD Guidelines for Testing of Chemicals. Organization for Economic Cooperation and Development, Paris.

Ramos, C., de la Torre, A.I., Tarazona, J.V., Muñoz, M.J., 1996. Desarrollo de un ensayo de inhibición de *Chlorella vulgaris* utilizando un test en microplacas. Revista de Toxicología 13, 97–100.

Repetto, G., Sanz, P., 1993. Neutral red uptake, cellular growth and lysosomal function: in vitro effects of 24 metals. Alternatives To Laboratory Animals 21, 501–507.

Repetto, G., Sanz, P., Repetto, M., 1993. In vitro effects of mercuric chloride and methylmercury chloride on neuroblastoma cells. Toxicology In Vitro 7, 353–357.

Repetto, G., Jos, A., Hazen, M.J., Molero, M.L., del Peso, A., Salguero, M., del Castillo, P., Rodríguez-Vicente, M.C., Repetto, M., 2001. A test battery for the ecotoxicological evaluation of pentachlorophenol. Toxicology In Vitro 15, 503–509.

Seiler, J.P., 2002. Pharmacodynamic activity of drugs and ecotoxicology -can the two be connected? Toxicology Letters 131, 105–115.

Sinues, B., Gazulla, J., Bernal, M.L., Lanuza, J., Fanlo, A., Saenz, M.A., Bartolomé, M., 1995. Six mutagenicity assays in exposure biomonitoring of patients receiving carbamazepine for epilepsy or trigeminal neuralgia. Mutation Research 334, 259–265.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Research 32, 3245–3260.

Title, T.V., Schaumann, B.A., 1992. Effect of antiepileptic drugs on growth of murine lymphoid tumor cells in single-cell culture. Epilepsia 33, 729–735.

Tjio, J.H., Levan, A., 1950. The use of oxiquinoline in chromosome analysis. Ann. Estac. Exp. Aula Dei 2, 21–64.

Vassault, A., 1983. Lactate dehydrogenase: UV method with pyruvate and NADH. In: Bergmeyer, H.V., Bergmeyer, J., Grabl, M. (Eds.), Methods of Enzymatic Analysis. Weinheim FRG Verlag Chemie, Weinheim, pp. 118–126.