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Biodegradation and Toxicity of the Antineoplastics Mitoxantron Hydrochloride and Treosulfane in the Closed Bottle Test (OECD 301 D)

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Hospital and communal waste water differ with respect to their content of specific chemical substances like disinfectants or medicinal drugs (Gartiser *et al.* 1996). Because the active substances of certain drugs are often metabolized poorly by patients after administration, they are excreted into waste water. Antineoplastic substances are supposed to be very stable (Allwood and Wright 1993; Lunn *et al.* 1989). Moreover, antineoplastics are known to be often carcinogenic, mutagenic, fetotoxic and embryotoxic (Allwood and Wright 1993). Till now, in the European Union (EU) medicaments need not be examined for their environmental properties: only a draft of a guideline exists, according which the examination of biodegradability is one of the important steps for evaluating the environmental impact of pharmaceuticals. Consequently, there are little information regarding their biodegradation in the aquatic environment. Traces of the antineoplastics ifosfamide and cyclophosphamide as well as other pharmaceuticals were detected in the sewage water of hospitals (Steger-Hartmann *et al.* 1996, Stan *et al.* 1994). Examination of their biodegradability with the Closed Bottle Test conducted previously showed that these antineoplastics are "not readily biodegradable" (Kümmerer *et al.* 1996).

We examined the biodegradability of other antineoplastics with different chemical structures and mechanisms of action than ifosfamide and cyclophosphamide, which alkylate after activation by eukaryotic cells: we studied the biodegradation of the intercalating anti-tumour agent mitoxantron hydrochloride and treosulfane, which alkylates without activation by eukaryotic cells using the Closed Bottle Test (OECD 301 D 1992). The testing of the biodegradation of these two antineoplastics is necessary to obtain knowledge about the possible risk for man and environment associated with these substances.

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MATERIALS AND METHODS

To prevent possible inhibitory and toxic effects of mitoxantron hydrochloride and treosulfane against the inoculum we carried out the growth inhibition test with *Pseudomonas putida* according to the German standard methods for the examination of water, waste water and sludge (DIN) with the modification of using a special nutrient medium for activated sludge bacteria (Brözel and Cloete 1992). The composition of this nutrient agar was previously described (Kümmerer *et al.* 1996).

To determine the biomass of *P. putida* we measured the optical density at 436 nm, and the protein concentration was quantified according to Lowry (Lowry *et al* 1951) with bovine serum albumin as external standard (Merck, Germany, order-no. 12018). The Closed Bottle Test was carried out with deionised water. Inhibitory effects of copper from the water used can be excluded, as the copper content was determined by atomic absorption spectrometry to be 1 µg/L. The inoculum was filtered (Schleicher & Schüll, Germany, order-no. 0851 1/2) and the first 200 mL were discarded (OECD 1992). The flasks were incubated in the dark at room temperature of 20±1 °C. The concentration of dissolved oxygen was measured by an oxygen electrode (Oxi 196; EO 196-1.5, WTW, Germany) according to German standard methods for the examination of water, waste water and sludge.

The Closed Bottle Test OECD 301 D was conducted according to the OECD guidelines (OECD 1992). Differing from the instructions, the test was prolonged to 40 days and the number of data points was increased to 11 to examine a possible adaptation of the bacteria on the tested substances. Mitoxantron hydrochloride was kindly provided by Cyanamid (Wolfratshausen, Germany; Batch-no. 331110) and treosulfane by Medac GmbH (Hamburg, Germany; Batch-no. 2055-07020). The concentration of the substances in the test was 5 mg/L.
The test consisted of the following different mixtures:
1. Test compound: mineral medium (M) + inoculum (I) + antineoplastic
2. Toxicity control: mineral medium (M) + inoculum (I) + sodium acetate + antineoplastic
3. Quality control: mineral medium (M) + inoculum (I) + sodium acetate
4. Blank: mineral medium (M) + inoculum (I).

At the same day the test was started, the inoculum was removed from the effluent of the communal waste water treatment plant of Forchheim (Abwasserzweckverband Breisgauer Bucht, Landkreis Emmendingen, Germany). In this communal sewage treatment plant the waste water of 6 different hospitals is biologically treated together with communal sewage.

Hospital effluent is about 1,5 percent of communal sewage. Degradation was monitored by analysis of dissolved oxygen over 40-d period. In addition, during the whole test period we determined the bacterial colony forming units (CFU) in all mixtures of the Closed Bottle Test using the same nutrient agar as in the inhibitory test with *P. putida* to verify a possible inhibition or toxicity on the inoculum by the test compounds. The CFUs give the number of active bacteria which grow on the nutrient agar used.

RESULTS AND DISCUSSION

A concentration of 10 mg/L mitoxantron hydrochloride caused a 99 percent growth inhibition of *P. putida* (IC_{100}), whereas treosulfane in a concentration up to 128 mg/L showed no inhibitory effect to *P. putida* (IC_0) (Fig. 3). The no inhibitory concentration of mitoxantron hydrochloride against *P. putida* (IC_0) was 5 mg/L. Krämer and Wenchel (1991) found, that mitoxantron hydrochloride up to a concentration of 9 mg/L has no inhibitory effect on *Pseudomonas aeroginosa.* However, until now there was no information available on the microbiological activity of mitoxantron hydrochloride against environmental bacteria (Bodet *et al.* 1985; Krämer and Wenchel 1991). The results of the inhibitory test with treosulfane confirm the results of biodegradation in the toxicity control. No data have been published so far concerning the microbiological stability and activity of treosulfane, because this antineoplastic is not often in use as yet (Sauer 1994).

Biodegradation of mitoxantron hydrochloride and treosulfane (Fig. 1 and Fig. 2), expressed as the percentage of ThOD (theoretical oxygen demand) is calculated by oxygen taken up by the microbial populations in the test vessel minus oxygen taken up by the blank. Fig. 1 and Fig. 2 show that both antineoplastics were not "readily biodegradable" according to the OECD guidelines (OECD 301 D 1992). Whereas there was no biodegradation of mitoxantron hydrochloride during the whole test period of 40 days, treosulfane was 30% biodegradable in the first 28 days of the test and 40% at the end of the test. An adaptation of the inoculum could be the reason for the increase of the biodegradation extent. The difference between the measured biodegradation of the mixture of antineoplastic and readily biodegradable sodium acetate (measured toxicity control) and the value of biodegradation computed from the biodegradation data of pure substances in test vessel and quality control never exceeded 25%. Consequently none of the tested substances had any toxic effect on the inoculum according to the test guidelines (Fig. 1 and Fig. 2).

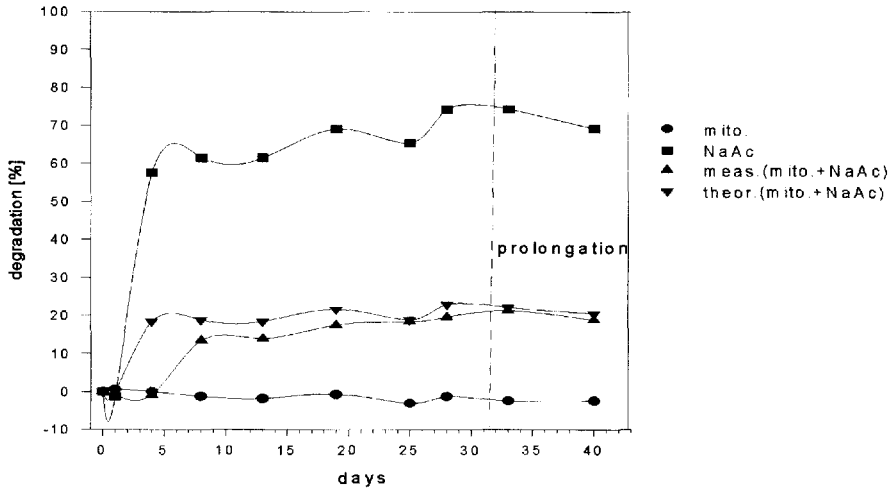

Figure 1. **Biological degradation of mitoxantron hydrochloride in the Closed Bottle Test. Negative values result from the computation procedure. Prolongation: Differing from the instructions the test was prolonged to 40 days. *mito.:* Biodegradation of mitoxantron hydrochloride in percent. *NaAc:* Biodegradation in percent for the reference compound sodium acetate, *meas.(mito. +NaAc):* Measured biodegradation of the mixture of mitoxantron hydrochloride and reference substance sodium acetate (toxicity control) in percent. *theor.(mito.+NaAc):* Computed biodegradation of the toxicity control mixture.**

Blok *et al.* (1985) studied the biodegradation of six different substances in six different tests. One of those tests was the Closed Bottle Test, in which the lowest ready biodegradability resulted. Therefore the Closed Bottle Test is often called a stringent test. The reason could be the low bacterial density in the test vessels. This encouraged us to determine the colony forming units during the test period although only a small part of the bacteria can be isolated from the effluent of the waste water treatment plant (Hiraishi *et al.* 1991). After eight days of the degradation test of mitoxantron hydrochloride the colony forming units were about 10-fold reduced compared to the blank. The toxicity control was comparable with the quality control (Fig. 4). Consequently an inhibitory effect of mitoxantron hydrochloride on the growth of the inoculum must be supposed. In the toxicity control with mitoxantron hydrochloride and sodium acetate this inhibitory effect determined by the colony forming units was absent, presumably because the conditions for the bacteria were changed by sodium acetate (Kümmerer *et al.* 1996). Thus these findings confirm that mitoxantron hydrochloride was not ready biodegradable. In the biodegradation test with treosulfane the colony forming units were nearly identical with the blank, whereas the toxicity

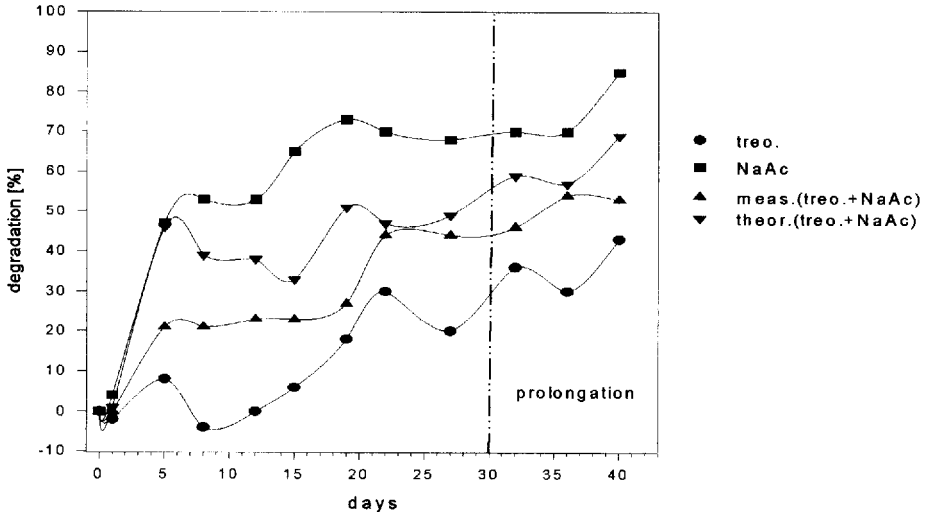

Figure 2. **Biological degradation of treosulfane in the Closed Bottle Test. Negative values result from the computation procedure. Prolongation: Differing from the instructions (OECD, 1992), the test was prolonged to 40 days. *treo.:* Biodegradation of treosulfane in percent. *NaAc:* Biodegradation of the reference substance sodium acetate in the quality control. *meas.* (treo.+NaAc): Measured biodegradation of a mixture of treosulfane and reference substance sodium acetate (toxicity control). *theor.* (treo.+NaAc): Computed biodegradation of the toxicity control mixture.**

control resulted in more CFUs than the blank. The number of CFUs in the toxicity control was nevertheless comparable with the quality control indicating no toxicity of treosulfane (Fig. 5).

Nyholm *et al.* (1991) assumed a loss of the inhibitory effect in the toxicity control previously but did not examine it in any more detail. Consequently we suppose that the toxicity control is not sufficient to detect the inhibitory effects of substances tested with the Closed Bottle Test. Thus we propose to use the additional determination of the colony forming units especially as a measurement for the inhibitory effect and as a supplement for the toxicity control of the Closed Bottle Test, when bactericidal substances are tested.

From an ecological point of view the better biodegradable treosulfane should be preferred to use compared with not biodegradable antineoplastics like cyclophosphamide, ifosfamide (Kümmerer *et al.* 1996) and mitoxantron hydrochloride, provided this is possible from the medical point of view.

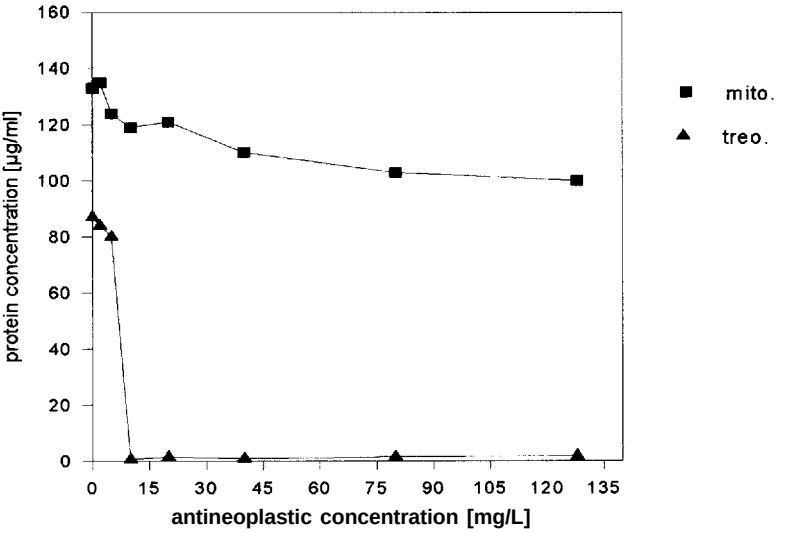

Figure 3. **Inhibitory test of mitoxantron hydrochloride and treosulfane against *Pseudomonas putida. mito.:* Inhibition by mitoxantron hydrochloride. *treo.:* Inhibition by treosulfane.**

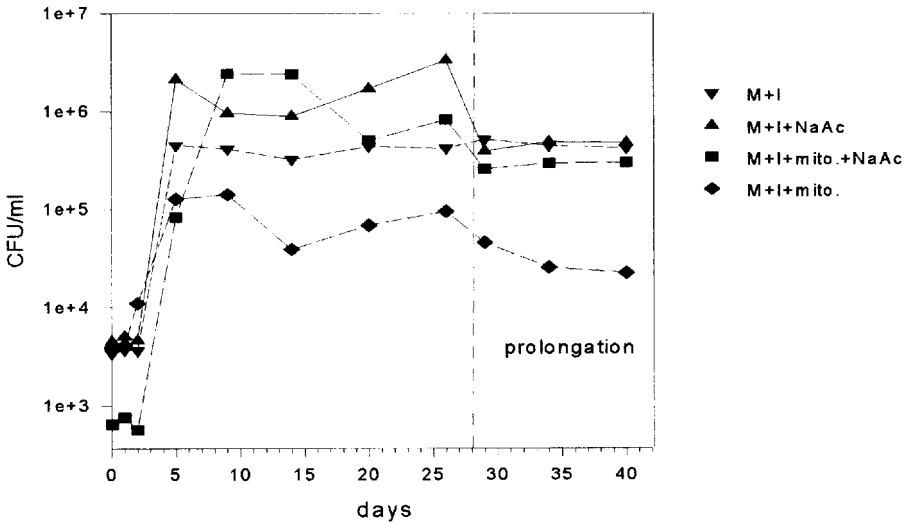

Figure 4. **Colony forming units in the degradation of mitoxantron hydrochloride (M+I+mito.), in the toxicity control (M+I+mito.+NaAc), in the blank (M+I) and in the quality control with sodium acetate (M+I+NaAc).**

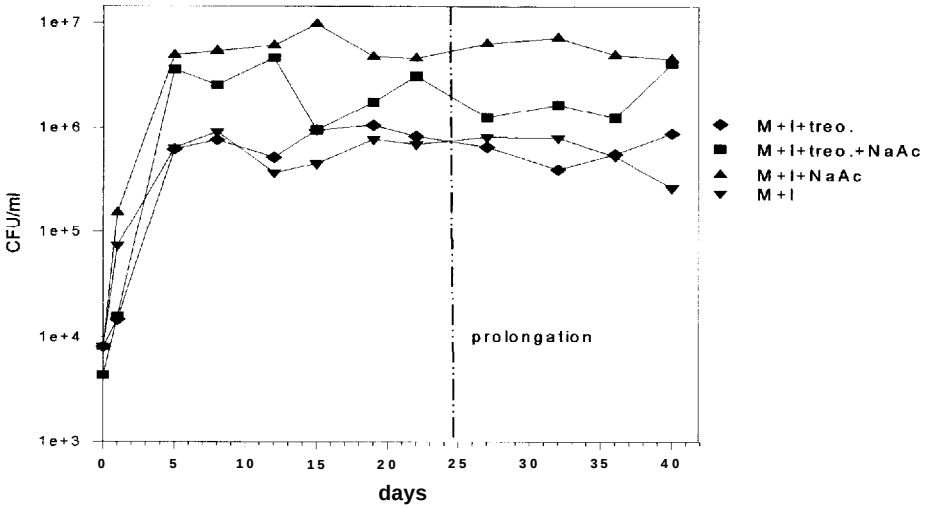

Figure 5. **Colony forming units in the degradation of treosulfane (M+l+treo.), in the toxicity control (M+l+treo.+NaAc), in the blank (M+l) and in the quality control with sodium acetate (M+l+NaAc).**

Investigations of biodegradability in other tests like the Zahn-Wellens Test (OECD 302 B 1992) are necessary to obtain better knowledge on the behaviour of mitoxantron hydrochloride and treosulfane in activated sludge and in the aquatic environment. Further examination of the elimination of mitoxantron hydrochloride and treosulfane in waste water treatment plants are necessary for a more complete knowledge of their environmental properties and to be able to draw further conclusions concerning the risk imposed by these substances onto the aquatic environment and man via drinking water.

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