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Development of an analytical method for the determination of anthracyclines in hospital effluents

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Abstract

Little is known about the fate of cytostatics after their elimination from humans into the environment. Being often very toxic compounds, their quantification in hospital effluents may be necessary to individualise the putative magnitude of pollution problems. We therefore developed a method for the determination of the very important group of anthracyclines (doxorubicin, epirubicin, and daunorubicin) in hospital effluents. Waste water samples were enriched by solid phase extraction (concentration factor 100), analysed by reversed-phase high performance liquid chromatography (RP-HPLC), and monitored by fluorescence detection. This method is reproducible and accurate within a range of 0.1–5 $\mu g\,l^{-1}$ for all compounds (limits of quantification: 0.26–0.29 $\mu g\,l^{-1}$; recoveries >80%). The applicability of the method was proven by chemical analysis of hospital sewage samples (range: 0.1–1.4 $\mu g\,l^{-1}$ epirubicin and 0.1–0.5 $\mu g\,l^{-1}$ doxorubicin). Obtained over a time period of one month, the results were in line with those calculated by an input–output model. These investigations show that the examined cytostatics are easily detectable and that the presented method is suitable to estimate the dimension of pharmaceutical contamination originating from hospital effluents.

Keywords: Doxorubicin; Epirubicin; Daunorubicin; Hospital effluents; RP-HPLC

1. Introduction

The therapeutic application of cytostatic agents in cancer therapy has been increasingly growing over the last years. As cytostatic agents act by either inhibiting cell growth or directly killing cells, many of them are known to be cytotoxic, mutagenic, and/or teratogenic. Due to the mentioned reaction mechanisms, cytostatics are likely to belong to the environmentally relevant compounds. The possible pollution of the environment can be attributed to different sources such as emissions from production sites or direct disposal of pharmaceuticals in households, but the main source of cytostatic compounds in waste water or in the environment are excretions (urine and faeces) of patients under medical treatment. In addition to the parent compound, active metabolites are excreted into sewage and may further enhance the cytotoxic potential of hospital effluents. Therefore, these substances may be detected in hospital sewer systems and even in the effluent of sewage treatment plants as they are probably not fully eliminated (Kümmerer, 1998). As cancer therapy is not only done in in-patient treatment wards but also in out-patient treatment wards (80% of all oncologic patients in the Vienna University Hospital), relevant amounts of cytostatic agents are expected to be detected in the public waste water. Despite these putative risks, little is known about the environmental fate of cytotoxic compounds. Moreover, for most of these agents the environmental burden is unknown, because the necessary analytical procedures have not been established

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yet. A few exceptions include the cytotoxics cyclophosphamide and ifosfamide (Steger-Hartmann et al., 1996; Steger-Hartmann et al., 1997; Kümmerer et al., 1997; Kiffmeyer et al., 1998).

We have recently shown that the analysis of cytotoxic agents such as 5-fluorouracil is feasible using capillary electrophoresis (Mahnik et al., 2004). Here, we present data on another group of anticancer agents, namely anthracyclines, using HPLC. The anthracyclines act to prevent cell division by disrupting structure and function of the DNA. They do so in two ways: (1) they intercalate into the base pairs in the DNA minor grooves; and (2) they cause free radical damage of the ribose in the DNA. Anthracyclines are frequently used in the treatment of haematological and solid neoplasms, including acute leukaemia, high-grade lymphoma, breast cancer, and bladder cancer (Oberdisse et al., 1997). These anthracyclines include daunorubicin, doxorubicin, and epirubicin (see Fig. 1).

The anthracycline doxorubicin is extracted from *Streptomyces peucetius var caesius*. It is the hydroxylated congener from daunorubicin. Epirubicin is an epimer from doxorubicin. The C4-hydroxylgroup is located in equatorial position instead of the axial position in doxorubicin (Dorr and Von Hoff, 1994).

Doxorubicin is administered in dosages ranging from 30 to 75 mg m^{-2} body surface, epirubicin in dosages ranging from 60 to 105 mg m^{-2} body surface and daunorubicin in dosages ranging from 40 to 60 mg m^{-2} body surface (Allwood et al., 1997). Approximately 3.5–5.7% of administered doxorubicin, 11% of epirubicin, and 13–15% of daunorubicin are excreted unmetabolised via urine. Metabolites include toxic compounds such as doxorubicinol, daunorubicinol or epirubicinol and non-toxic agents such as 7-hydroxy and 7-deoxy aglycones (Dorr and Von Hoff, 1994). The urinary excretion of anthracycline metabolites amounts to 0.7–4.3% doxorubicinol, 0.35–1.76% epirubicinol, 0.4% epirubicinol glucuronides and 3.3% epirubicin glucuronide (Camaggi et al., 1988; Mross et al., 1988). The therapeutic potential of doxorubicin is limited by its cardiotoxicity. Since the major metabolite doxorubicinol is up to 10 times more potent than doxorubicin, this metabolite is supposed to be involved in causing cardiotoxicity (Boucek et al., 1987; Olson et al., 1988). From the IARC (International agency on the research of cancer) doxorubicin and daunorubicin are classified as category 2 (A and B), whereby 2A means probably carcinogenic and 2B means possibly carcinogenic.

Biological degradation of the anthracyclines has been reported for epirubicin only, but not for doxorubicin and daunorubicin. The cytostatic compound epirubicin was investigated in the closed-bottle-test (OECD 301D). At concentrations of 5 mg l^{-1} no degradation could be observed. In contrast to the closed-bottle-test, the Zahn-Wellens-test (OECD 302B) is carried out with a higher concentration of bacteria and a higher concentration of test substance. Tested under these conditions, epirubicin was eliminated, but mainly by adsorption to sewage sludge. The substance showed nearly no toxicity against bacteria in the closed-bottle-test, whereas in the Zahn-Wellens-test some adverse effects could be detected (Kümmerer et al., 1996; Kümmerer, 1999). It has to be mentioned, however, that the concentrations used for these tests were much higher (10^{-6}–10^{-7}) than the concentrations expected in the waste water of hospitals. Due to the high adsorption tendency of epirubicin, neither biodegradation in sewage treatment plants nor emission to surface water is supposed as anthracyclines readily adsorb to glass, steel, and variable synthetic material including polypropylene, polytetrafluorethylene, and polyethylene.

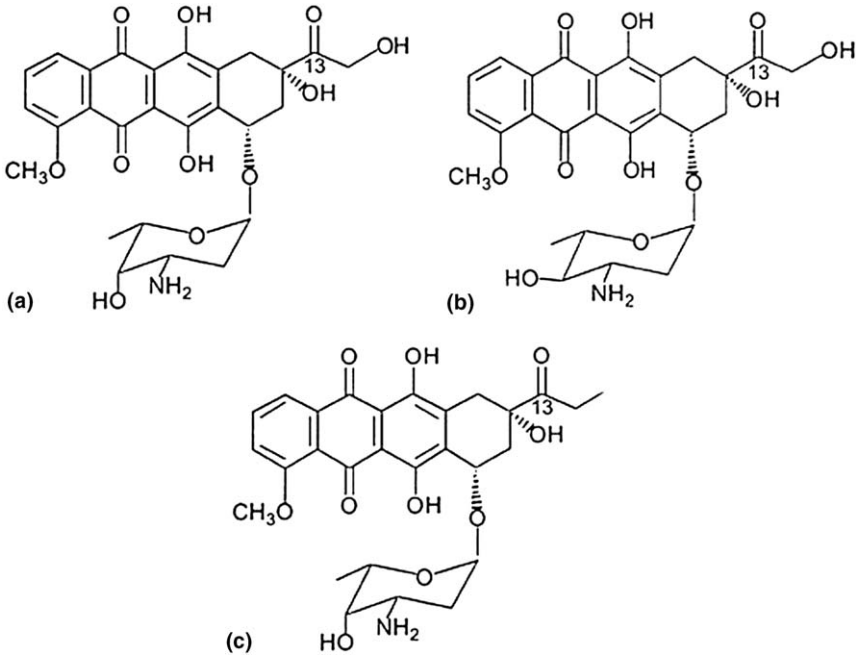

Fig. 1. (a) Structural formula of doxorubicin, (b) structural formula of epirubicin, and (c) structural formula of daunorubicin.

In fact, anthracyclines are expected to be removed from waste water together with sewage sludge (Kümmerer, 1999).

To estimate possible concentrations of cytostatic substances in hospital effluents, an input–output model has been established, based on the parameters dosage, consumption, excretion rate, and water consumption. To provide an idea of the administered amounts of anthracyclines, in the Vienna University Hospital approximately 0.1 kg doxorubicin, 0.13 kg epirubicin and 0.02 kg daunorubicin are consumed every year (Mahnik et al., 2003). According to this model, a concentration range of 0.03–2.7 $\mu g\,l^{-1}$ has to be expected for the cytostatic agents doxorubicin, epirubicin, and daunorubicin.

Here, we present a method for the reliable detection of low concentrations of doxorubicin, epirubicin, and daunorubicin using solid phase extraction and RP-HPLC.

2. Materials and methods

2.1. Chemicals and equipment

Epirubicin was purchased from Ebewe (Unterach, Austria), doxorubicin and daunorubicin from Pharmacia Upjohn (New York, USA). Water and acetonitrile (ACN) were of HPLC quality (Merck, Darmstadt, Germany), methanol, chloroform, *n*-hexane, sodium hydroxide, and *ortho*-phosphoric acid were of p.a. quality (Merck). Bovine serum albumin (BSA) was from Merck, whereas phosphate buffered saline (PBS) was purchased from GIBCO (Paisley, UK).

All centrifuge tubes used and HPLC vials were made of polypropylene to minimize the adsorption effects of anthracyclines. For solid phase extraction, C8 columns (500 mg/3 ml) from Varian (CA, USA) were used.

A 1090-HPLC from Agilent Technologies (Waldbronn, Germany) with a C18 column (Hypersil ODS, octadecyl silane monolayer, endcapped; particle size: 5 μm, pore size: 120 Å, column dimension: 2.1×200 mm) from Hewlett Packard and a pre-column from Phenomenex (octadecyl, 4 mm × 3.0 mm C18 ODS) was used. The fluorescence of doxorubicin, epirubicin, and daunorubicin was monitored using a fluorescence detector (excitation wavelength: 480 nm, emission wavelength: 560 nm).

2.2. Sample enrichment

It is known, that anthracyclines readily adsorb to matrices such as plastic and glass leading to irreproducible analytical results. As standard curves in water did not run linear, we hypothesised that addition of bovine serum albumin (BSA) would result in linear standard curves as known for the matrix plasma. Adding BSA (2%), the high protein binding capacity (>70% anthracyclines adsorbed) is exploited (Dorr and Von Hoff, 1994).

To ascertain the absence of cytostatic compounds, waste water blanks of industrial origin were obtained from a sequencing batch reactor of the University of Natural Resources and Applied Life Sciences. Ten milliliters of each sample were centrifuged 10 min at 5000*g* and the eluate filtered through sterile filters (Corning Top Filter, New York, USA) with 0.22 μm pore size and a cellulose acetate membrane to remove suspended solids. For quality control testing, we spiked sewage water with different concentrations of doxorubicin, epirubicin, and daunorubicin using a stock solution of 1 $mg\,ml^{-1}$ of doxorubicin, epirubicin, and daunorubicin in DMSO/0.9% physiological NaCl solution (1:1, v/v) to obtain the following concentrations: 0.1 $\mu g\,l^{-1}$, 0.5 $\mu g\,l^{-1}$, 1 $\mu g\,l^{-1}$ and 5 $\mu g\,l^{-1}$ in waste water. The pH of the samples was adjusted by NaOH to 7.5. The C8 columns were preconditioned by 5 ml MeOH, 5 ml water and 5 ml PBS containing 2% BSA (w/v). The sorbent should not be allowed to become desolvated by excessive drying, especially before applying the sample. The preconcentration of the analytes was carried out using a commercially available apparatus (VacElut Analytichem, VacMaster IST). The samples were applied to the columns without any pressure (flow rate approximately 2 $ml\,min^{-1}$). After enrichment, the columns were washed with 5 ml water and residual water was displaced by applying 5 ml *n*-hexane. Doxorubicin, epirubicin, and daunorubicin were eluted with 1.5 ml MeOH/$CHCl_3$ (1:2, v/v). The solvent of the eluate was evaporated to dryness and the residue dissolved in 100 μl 30% ACN/70% K-di-hydrogenphosphate buffer (pH = 2) in order to avoid loss of the analytes by surface interaction (Rudolfi et al., 1995). For further clean up, this extract was centrifuged 2 min at 21000*g* and the eluate transferred to HPLC vials.

2.3. Analytical parameters

The conditions for HPLC analysis were as follows: Samples were automatically injected with a 25 μl syringe (injection volume: 20 μl) and the analytes were separated on a reversed-phase C18 analytical column. Separation was carried out using a binary gradient with a stepwise increasing percentage of acetonitrile. For the aqueous part of the eluent, the pH of a 10 mM K-di-hydrogenphosphate buffer was adjusted to 2.0 (Rudolfi et al., 1995). Solvents and gradients are shown in Table 1. The flow rate was set to

Table 1
HPLC parameters

Column	Reversed-phase C18 analytical column, (Hypersil ODS, 5 μm, 2.1×200 mm), Hewlett Packard		
Eluent	Buffer A: 10 mM K-di-hydrogenphosphate buffer, pH = 2 Buffer B: acetonitrile		
Gradient	Time (min)	Buffer A (%)	Buffer B (%)
	0	90	10
	1	90	10
	21	50	50
	31	0	100
	41	90	10
	51	90	10

0.6 ml min^{-1}. Substances were identified by their retention time in the fluorescence scan and quantified by peak area.

Under these conditions, doxorubicin, epirubicin, and daunorubicin were monitored by fluorescence with a typical retention time between 13 and 16 min.

3. Results and discussion

3.1. Method development

From our own prior studies it is known that standard curves of anthracyclines in plasma are linear, as opposed to standard curves in water. To minimize adsorption effects on container surfaces when carrying out a solid phase extraction, waste water samples were spiked with BSA (2%) exploiting the high protein binding capacity of the anthracyclines ($\geqslant$70% anthracyclines adsorbed).

Validation of this method has been carried out according to the guidelines published by the Journal of Chromatography B (Lindner and Wainer, 1996) and the Note for Guidance on validation of analytical procedures (EMEA, 1996).

3.2. Quality control

The standard curves were linear up to 5 μg l^{-1} doxorubicin, epirubicin, and daunorubicin in waste water, when assaying five calibration standards. Each calibrator was injected five times. Calculating a linear regression, the coefficient of correlation was 0.9995 for doxorubicin, 0.9996 for epirubicin and 0.9993 for daunorubicin (see Fig. 2).

Comparing the calibration standards dissolved in PBS/BSA (2%) with that in waste water, the mean overall recovery was 85.2% for doxorubicin, 86% for epirubicin and 87.6% for daunorubicin. The relative standard deviation of the recoveries covering four different concentration levels was below 10%, with the exception of epirubicin (12%). The recoveries of the single calibrators are shown in Table 2. The applicability of the presented method to all anthracyclines precluded the use of an internal standard such as idarubicin.

Table 2
Recovery of doxorubicin, epirubicin, and daunorubicin ($n = 5$)

Concentration in waste water [μg l^{-1}]	Doxorubicin mean value [%]	Epirubicin mean value [%]	Daunorubicin mean value [%]
0.1	86.9	88.4	85.3
0.5	79.8	83.7	89
1	84.1	84.3	85.2
5	89.9	87.5	90.8

The repeatability of the method was estimated by evaluation of the calibration standards within a sequence (within-day precision, $n = 5$) and on five consecutive days (between-day precision). The relative standard deviation was below 9% for doxorubicin, below 12% for epirubicin and below 10% for daunorubicin (see Table 3).

The accuracy of the method was proven by analysis of 3 concentrations of every substance ($n = 3$). The mean overall accuracy of the method was −3.2% deviation from the target value for doxorubicin, −4.6% for epirubicin, and −7.9% for daunorubicin (see Table 4).

Considering a signal to noise ratio of 3:1, the limit of detection was calculated to be 0.05 μg l^{-1} doxorubicin, 0.05 μg l^{-1} epirubicin, and 0.06 μg l^{-1} daunorubicin in waste water. The quantification limit was calculated to be 0.26 μg l^{-1} doxorubicin, 0.26 μg l^{-1} epirubicin, and 0.29 μg l^{-1} daunorubicin in waste water (signal to noise ratio 15:1).

When assaying waste water samples from the sewer of the oncologic in-patient treatment ward no interferences were observed. The standard curves in waste water were all linear with a coefficient of correlation of 0.9995 for

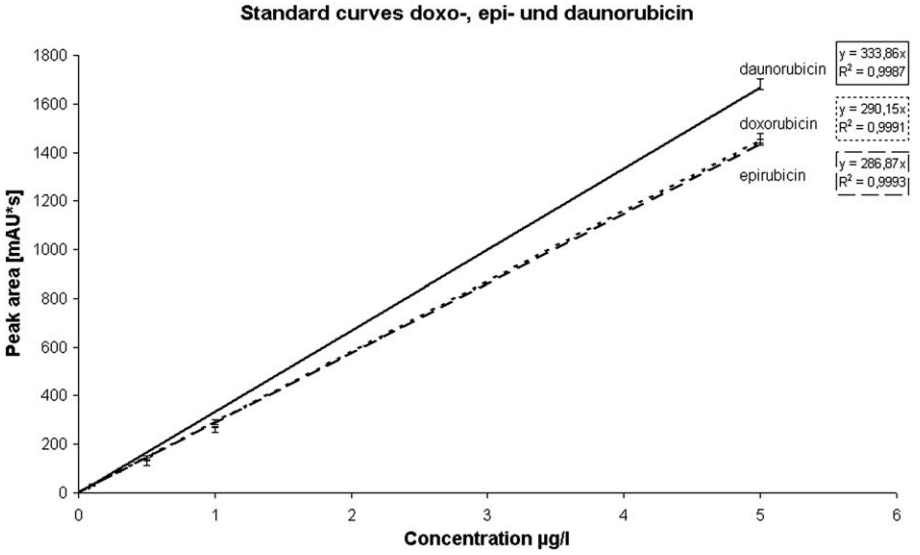

Fig. 2. Linearity of the standard curves for doxorubicin, epirubicin, and daunorubicin in waste water.

Table 3
Repeatability of the analytical method ($n = 5$) for doxorubicin, epirubicin, and daunorubicin

Concentration in waste water [$\mu g\,l^{-1}$]	Doxorubicin		Epirubicin		Daunorubicin	
	Within-day precision (relative standard deviation) [%]	Between-day precision (relative standard deviation) [%]	Within-day precision (relative standard deviation) [%]	Between-day precision (relative standard deviation) [%]	Within-day precision (relative standard deviation) [%]	Between-day precision (relative standard deviation) [%]
0.1	3.5	6.8	7.6	10.4	7.5	7.7
0.5	8.7	9.5	11.4	12.1	10.3	9.3
1	4.9	4.2	4	8.4	3.6	8.1
5	1.3	8.2	0.9	10.6	0.7	8.8

Table 4
Accuracy of the analytical method ($n = 5$) for doxorubicin, epirubicin, and daunorubicin

Concentration in waste water [$\mu g\,l^{-1}$]	Doxorubicin		Epirubicin		Daunorubicin	
	Mean result of the analysis [$\mu g\,l^{-1}$]	Accuracy [%]	Mean result of the analysis [$\mu g\,l^{-1}$]	Accuracy [%]	Mean result of the analysis [$\mu g\,l^{-1}$]	Accuracy [%]
0.5	0.47	−6.04	0.47	−6.21	0.45	−9.56
2	1.91	−4.57	1.84	−8.18	1.77	−11.57
5	5.05	0.97	5.02	0.40	4.87	−2.7

doxorubicin, 0.9996 for epirubicin, and 0.9993 for daunorubicin (see Fig. 2). Together with the quality control data, this strongly suggests this method to be specific for doxorubicin, epirubicin, and daunorubicin.

3.3. Application and limits of the analytical method

This method has been validated to detect doxorubicin, epirubicin, and daunorubicin in hospital effluents at concentrations of 0.1–5 $\mu g\,l^{-1}$. Using this method, we were able to determine epirubicin and doxorubicin in waste water samples of the Vienna University Hospital at concentrations of 0.1–1.4 $\mu g\,l^{-1}$ epirubicin and 0.1–0.5 $\mu g\,l^{-1}$ doxorubicin when monitoring the sewer system of the oncologic in-patient treatment ward for 28 days. The sewer system of the oncologic in-patient treatment ward allowed for the collection of waste water from 18 oncologic patients in two 1000 l storage tanks over a time period of 24 h. After blending the tank for 15 min, waste water samples representing a 24-h collection period were taken. During the monitoring period, daunorubicin could not be detected, because the substance was not administered to patients. The applicability of the presented method is illustrated by chromatograms of spiked samples and hospital waste water samples (Figs. 3 and 4, respectively). The results of the chemical analysis were in line with the data calculated by an input–output model, which was carried out prior to monitoring (calculated range of anthracyclines: 0.03–2.7 $\mu g\,l^{-1}$). Briefly, the range of expected concentration has been calculated from the parameters drug consump-

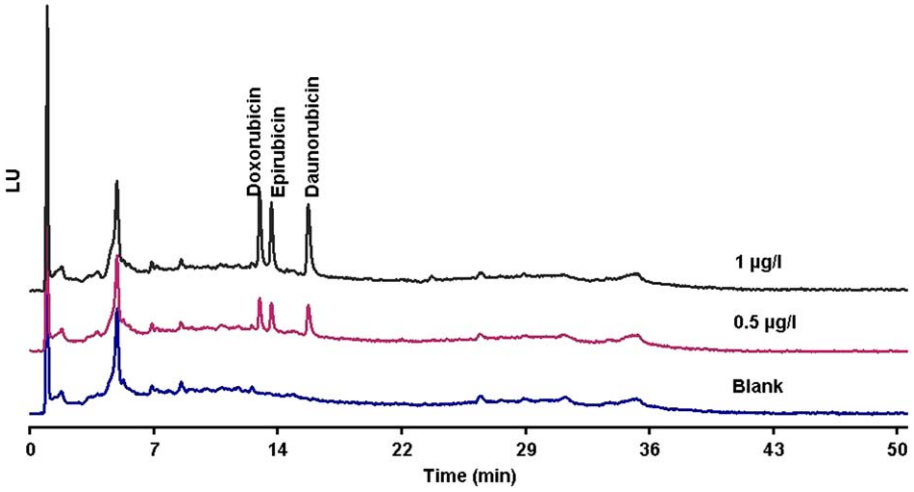

Fig. 3. Chromatogram of waste water samples. Waste water blank vs. waste water spiked with doxorubicin, epirubicin, and daunorubicin.

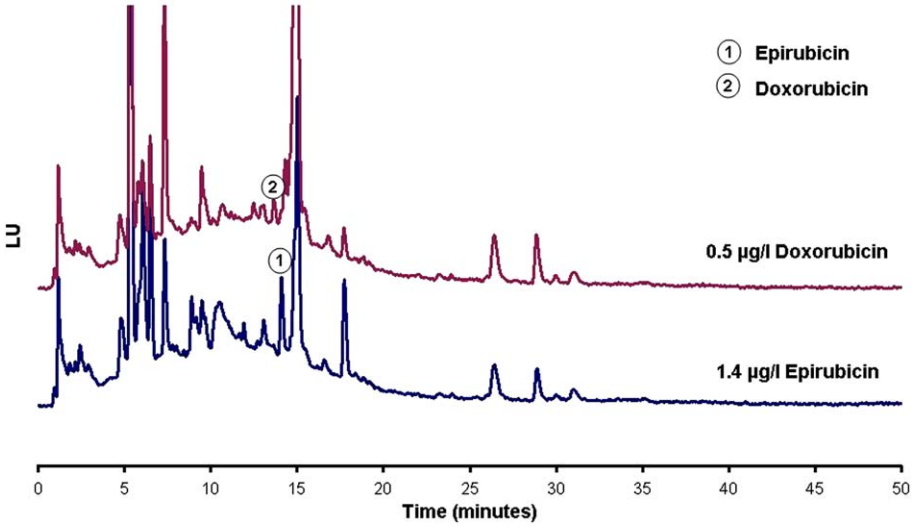

Fig. 4. Chromatogram of waste water samples of the oncologic sewer system in the Vienna University Hospital. Waste water samples from the sewer system of the oncologic in patient treatment ward analysed by the described method.

tion, human excretion rate and water consumption, as described in detail previously (Mahnik et al., 2003). The method, however, is not sensitive enough to assess cytostatics in the wastewater of the entire hospital including the sewage from all non-oncologic departments. Since from our data it is clear that elimination via wastewater occurs, even minute amounts of a CMR (cancerostatic, mutagenic and reprotoxic) agent have to be considered an environmental burden. Due to the lack of analytical assessments in this extremely low concentration range, the real extent of this burden remains to be determined.

The excretions of patients receiving their therapy on an out-patient base should not differ significantly from those of an in-patient ward. However, all data reported in this manuscript refer to a central hospital and a central collection covering only the excreta of several patients under chemotherapy. Very likely, this situation is not directly comparable to that of single patients leaving the hospital immediately after therapy.

In agreement with our input–output model, we have recently shown that 5-fluorouracil can be detected in much higher levels (up to 122 $\mu g\,l^{-1}$) in hospital effluents when compared with anthracyclines, mainly due to the higher administered dosage and higher excretion rates (Mahnik et al., 2004). Nevertheless, the toxicity of anthracyclines is classified as possibly carcinogenic for doxorubicin and probably carcinogenic for daunorubicin by the IARC (International agency on the research of cancer). In contrast, 5-fluorouracil is not classifiable as a carcinogenic, i.e. the data available is not sufficient for a final classification of the compound. As a consequence, even small concentrations of anthracyclines in waste water may be of concern. Moreover, other putative risk factors such as chronic effects of the compounds, of their metabolites or synergistic effects with other substances or pharmaceuticals in waste water have not been characterised yet. This may be particularly relevant for hospital effluents, which usually contain a wide spectrum of chemical substances and pharmaceuticals. Up to 4.3% and 1.76% of the consumed amount of anthracyclines are excreted via urine in form of the toxic metabolites doxorubicinol and epirubicinol. Considering that the parent anthracyclines are excreted up to 15% via urine, the metabolites are likely to represent a relevant contribution to wastewater toxicity.

Even, if the expected concentrations in hospital effluents are low compared with other antineoplastic agents, further investigations on the occurrence and fate of anthracyclines may be of relevance because of their carcinogenic potential for humans and the environment.

4. Conclusions

Our results show that the presented method allows for the analysis of the anticancer agents doxorubicin, epirubicin, and daunorubicin in hospital effluents at low concentrations (down to 0.1 $\mu g\,l^{-1}$ of each compound). The observed monitoring data in waste water are in line with the results of the input–output model calculation and estimations found in the literature. Therefore, this method is suitable to determine the dimension of pharmaceutical contamination originating from hospitals effluents. Once identified as a putative environmental risk, further investigations about the consequences of chronic exposure and synergistic effects with other pharmaceuticals need to be carried out.

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