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Multianalyte determination of 24 cytostatics and metabolites by liquid chromatography–electrospray–tandem mass spectrometry and study of their stability and optimum storage conditions in aqueous solution

Noelia Negreira [a], Nicola Mastroianni [a], Miren López de Alda [a,*], Damià Barceló [a,b]

[a] Water and Soil Quality Research Group, Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA-CSIC), Jordi Girona 18-26, E-08034 Barcelona, Spain

[b] Catalan Institute for Water Research (ICRA), H_2O Building, Scientific and Technological Park of the University of Girona, Emili Grahit 101, 17003 Girona, Spain

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ABSTRACT

A multianalyte liquid chromatography–electrospray–tandem mass spectrometry (LC–ESI–MS/MS) method for determination of 19 cytostatics and 5 metabolites, from 6 different therapeutic families, has been developed, and the structures of the main characteristic fragment ions have been proposed. Instrumental limits of detection and quantification are in the range 0.1–10.3 and 1.0–34.3 ng mL^{-1}, respectively. Moreover, the stability of the compounds in aqueous solution was investigated in order to establish the best conditions for preparation and storage of both calibration standards and water samples. Dimethylsulphoxide (DMSO) was selected as solvent for preparation of the stock solutions. At room temperature (25 °C), 11 of the 24 target compounds were shown to be unstable in water (percentage of organic solvent 4%), with concentration losses greater than 20% in less than 24 h. At 4 °C (typical storage temperature for water samples) all compounds, except MTIC and chlorambucil, were stable for 24 h, but the number of stable compounds decreased to 10 after 9 days. Freezing of the aqueous solutions improved considerably the stability of various compounds: after 3 months of storage at −20 °C, 10 compounds, namely, 5-fluorouracil, carboplatin, gemcitabine, temozolomide, vincristine, vinorelbine, ifosfamide, cyclophosphamide, etoposide, and capecitabine, remained stable (in contrast to only carboplatin and capecitabine at 4 °C). The addition of acid improved the stability of methotrexate and its metabolite hydroxy-methotrexate but not that of the rest of compounds. The addition of organic solvent (50% methanol or DMSO) prevented the degradation at 4 °C of the otherwise unstable compounds oxaliplatin, methotrexate, erlotinib, doxorubicin, tamoxifen, and paclitaxel. To the authors' knowledge, five of the analytes investigated have never been searched for in the aquatic environment (imatinib, 6α-hydroxypaclitaxel, endoxifen, (Z)4-hydroxytamoxifen, and temozolomide), and for many of them the stability data provided, and even the analytical LC–MS/MS conditions, are the first ever published.

1. Introduction

During recent years great consideration has been given to the contamination of the environment by veterinary and human pharmaceuticals. Several groups of pharmaceuticals, such as antibiotics and hormones, have been studied intensively; however, other potentially more toxic compounds, such as cytostatic agents, have received very little attention [1].

The use of cytostatics for cancer therapy has increased considerably in the last decade [2,3], and their production has been estimated to be 5000 kg per year [4]. These substances act by either inhibiting cell growth or directly killing cells (cytotoxic) [4,5]. The reaction mechanisms of cytostatic compounds and their mainly non-specific way of attack within organisms often give rise to secondary side effects and increased health risks [4]. Thus, many antineoplastic agents have cytotoxic, mutagenic, carcinogenic, embryotoxic and/or teratogenic effects [1,4–6]. Recent studies have reported that persons working with cytostatic drugs, e.g. clinical and pharmaceutical staff, show a marginally higher frequency of DNA damage. Moreover, they can be introduced in the environment, and even enter the food chain, through excretion from patients under medical treatment as main source [5,7]. In fact, some of them have been detected in hospital wastewater at concentration levels varying from ng L^{-1} to μg L^{-1} [8,9].

* Corresponding author. Tel.: +34 93 4006100; fax: +34 93 2045904.
E-mail address: mlaqam@cid.csic.es (M. López de Alda).

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5-Fluorouracil (5-Fu), ifosfamide (IF), and cyclophosphamide (CP) have been shown to be the most abundant compounds [8,10–12], but they have been also the most investigated cytostatics. A few works have addressed the study of doxorubicin (DOX), vincristine (VCN), etoposide (ETP), and methotrexate (MET) [2,10,13], reporting their occurrence at low concentrations [8]. Martin et al. [14] analyzed for the first time some cytostatics not included in previous works, namely, paclitaxel (PAC), irinotecan (IRI), vinorelbine (VRB), and gemcitabine (GEM), but they were either not detected (PAC and IRI) or detected at low levels (lower than 10 ng L^{-1} in influent wastewater) (VRB and GEM). Recently, the application, in a second-phase of the present work, of a newly developed on-line solid phase extraction (SPE)–LC–MS/MS method to the analysis of 13 cytostatics and 4 metabolites in waters, has expanded the list of anti-cancer drugs measured in environmental samples to 8 new compounds (temozolomide (TMZ), imatinib (IMA), erlotinib (ERL), capecitabine (CAP), hydroxytamoxifen (OH-TAM), endoxifen or 4-hydroxy-N-desmethyl-tamoxifen (OH-D-TAM), and hydroxypaclitaxel (OH-PAC)), and has evidenced for the first time the presence of CAP and OH-PAC in influent wastewaters at levels up to 30 ng L^{-1} [15]. Carboplatin has also been detected in a hospital effluent [7] and, to the authors' knowledge, other compounds have never been studied in water samples.

On the other hand, some experimental studies [1,16–19] have reported genotoxicity in hospital wastewater effluent samples collected both before and after treatment in wastewater treatment plants (WWTPs), but the genotoxicity of pure compounds with ecotoxicological bioassays has been investigated in only a few occasions [16,20].

Most cytostatics are polar compounds often having a high molecular weight, circumstances that limit the use of gas chromatography (GC) techniques for their determination since a derivatization step is usually necessary to enhance the volatility and the thermal stability of the compounds before their injection into the chromatographic system. Thus, only 5-Fu, TAM, IF and CP have been determined by GC coupled to mass spectrometry (MS) [11,21,22]. Liquid chromatography (LC) coupled to ultraviolet (UV) detection has been commonly employed for determination of cytostatics [4,8,23]. However, trace-level environmental analysis requires higher sensitivity, specificity and accuracy, which are only possible using MS detection. The application of this advanced technique to cytostatics analysis has been described in some recent works [6,12,14,24–26]. However, many of them focus on the analysis of just a few classes of cytostatics, and structural information on the product ions chosen for selected reaction monitoring (SRM) determination has been often overlooked. Only Gómez-Canela et al. [27] have provided mass spectral characterization for 26 cytostatics, 14 of which are measured in the present study (all but TMZ, 5-(3-N-methyltriazen-1-yl)-imidazole-4-carboxamide (MTIC), MET, 5-Fu, VRB, carboplatin (Carb-Pt), Oxaliplatin (Oxa-Pt) and the metabolites OH-MET, OH-PAC, OH-D-TAM and OH-TAM).

The number of studies investigating and reporting the presence of cytostatics in water samples is quite short. This fact can be attributed to either a low medical use or, most probably, to the existence of degradation processes in the aqueous medium leading to the transformation of the active principles into other products, which, in turn, can be more toxic and persistent than the original ones. Little information is known in this respect, even though some biodegradation products have already been identified. For example, the biodegradation process of MET is combined with the generation of the toxic and persistent degradation product 7-hydroxymethotrexate (OH-MET) [4], TMZ decomposes to MTIC in the DNA at pH > 7 [28], PAC to OH-PAC, and tamoxifen (TAM) to OH-TAM and OH-D-TAM. Moreover, low recoveries in the analysis of some cytostatics in aqueous samples have been reported by some authors [11,14,29], which, in fact, could have their origin in the possible instability of the compounds in the samples.

In this context, the objectives of this work were: (1) to explore the possibilities of LC–ESI–MS/MS for the analysis of cytostatics by optimizing a multi-analyte method for the simultaneous determination of 19 anti-cancer drugs and 5 metabolites belonging to different Anatomical Therapeutic Classification (ATC) classes; (2) to provide information on their fragmentation pattern, an aspect overlooked in the literature; and (3) to study their stability and optimum storage conditions in aqueous solution.

2. Experimental

2.1. Standards and solvents

All solvents were of HPLC grade and all chemicals were of analytical reagent grade. Formic acid (98–100%), ammonium hydroxide (25%), methanol and HPLC-water were purchased from Merck (Darmstadt, Germany), while DMSO (DMSO, > 99.9%) was acquired from Aldrich (Milwaukee, WI, USA).

Analytical standards of the cytostatic compounds CAP, Car-Pt, chlorambucil (CHL), CP, DOX hydrochloride, ERL hydrochloride, ETP, 5-Fu, GEM hydrochloride, IF, IMA mesylate, MET, OH-MET, MTIC, Oxa-Pt, 6(α)–OH-PAC, TAM citrate, (Z)-4-OH-TAM, OH-D-TAM, TMZ, vinblastine (VBL) sulphate, VCN sulphate, and VRB ditartrate were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Paclitaxel (PAC) was supplied by Sigma-Aldrich at the highest available purity (> 99%).

The selected cytostatics, grouped into six families attending to their mode of action and chemical structure, are shown in Table 1.

Individual solutions of each compound (ca. 1000 μg mL^{-1}) and a mixture of them (ca. 25 μg mL^{-1}) were prepared in DMSO and stored in the dark at −20 °C.

Different working standard solutions were made by appropriate dilution in ultrapure water, methanol, DMSO or mixtures of them, and were then immediately analyzed by LC–MS/MS.

2.2. Safety considerations on cytostatic drugs handling

As cytostatic drugs are highly toxic compounds, their handling requires strict safety precautions in order to guarantee the best-possible protection of research workers. All stock solutions were prepared under a biological safety hood with laminar airflow and absorbent paper was used to protect the work surfaces. All instruments and materials that were in contact with tested compounds were disposable and treated as a hazardous waste.

2.3. Instrumental and chromatographic conditions

Analyses were carried out using an Acquity UPLC system (Waters, Milford, MA, USA) consisting of a thermostated autosampler, a binary pump, a vacuum degasser, a thermostated column compartment, and a UV–vis programmable detector. The UPLC system was coupled to a Waters TQD triple quadrupole (QqQ) mass spectrometer equipped with an electrospray ionization (ESI) source. The whole LC–MS/MS system was controlled by MassLynx 4.1 software (Waters).

Chromatographic separation of the cytostatic drugs was performed on a reversed-phase column Purospher STAR RP-18e (125 × 2 mm, 5 μm particle size) from Merck, maintained at 25 °C. Ultrapure water (A) and methanol (B), without modifier or containing 0.1% of formic acid, were tested as mobile phases. Under final optimized conditions, compounds were separated with the acidified mobile phase using the following gradient: 0–1 min, 5% B; 2 min, 20% B; 12 min, 80% B; 19–23 min, 100% B;

Table 1
Abbreviated names, molecular mass, acid–base dissociation constant (pKa), octanol–water partition coefficient (log K_{ow}), and ATC group of the target cytostatics.

Compound (acronym)	Molecular mass	pKa[b]	Log K_{ow}[b]	Group
Alkylating agents				
Cyclophosphamide (CP)	260.02	2.84	0.73	Nitrogen mustard analogues
Ifosfamide (IF)	260.02	1.44	0.78	Nitrogen mustard analogues
Chlorambucil (CHL)	304.21	4.82 (acidic) 4.62 (basic)	2.61	Nitrogen mustard analogues
Temozolomide (TMZ)	194.20	14.77 (acidic) −1.63 (basic)	−1.27	Other alkylating agents
MTIC[a]	168.16	10.07 (acidic) 2.23 (basic)	−1.16	Other alkylating agents
Antimetabolites				
Methotrexate (MET)	454.45	3.47 (acidic) 5.56 (basic)	−0.45	Folic acid analogues
Hydroxymethotrexate (OH-MET)[a]	470.44	3.48 (acidic) 4.99 (basic)	−0.69	Folic acid analogues
5-Fluorouracil (5-FU)	130.02	–	−0.65	Pyrimidine analogues
Gemcitabine (GEM)	263.20	11.65 (acidic) 4.26 (basic)	−2.22	Pyrimidine analogues
Capecitabine (CAP)	359.15	5.41 (acidic) 1.75 (basic)	1.04	Pyrimidine analogues
Plant alkaloids and other natural products				
Vinblastine (VBL)	810.97	11.36 (acidic) 7.90 (basic)	5.92	Vinca alkaloids and analogues
Vincristine (VCN)	824.96	11.10 (acidic) 7.90 (basic)	5.75	Vinca alkaloids and analogues
Vinorelbine (VRB)	778.93	11.36 (acidic) 6.90 (basic)	7.08	Vinca alkaloids and analogues
Etoposide (ETP)	588.57	9.94 ± 0.40 (acidic)	0.28	Podophyllotoxin derivatives
Paclitaxel (PAC)	853.91	11.90 (acidic) −2.19 (basic)	3.95	Taxane
6(α)-Hydroxypaclitaxel (OH-PAC)[a]	869.91	11.90 (acidic) −2.19 (basic)	3.19	Taxane
Cytotoxic antibiotics and related substances				
Doxorubicin (DOX)	543.52	7.35 (acidic) 8.68 (basic)	1.27	Anthracyclines and related substances
Other antineoplastic agents				
Carboplatin (Car-Pt)	371.25	–	−2.19	Platinum compounds
Oxaliplatin (Oxa-Pt)	397.29	–	−1.67	Platinum compounds
Imatinib (IMA)	493.60	13.28 (acidic) 7.55 (basic)	2.89	Protein kinase inhibitors
Erlotinib (ERL)	393.44	5.32	3.03	Protein kinase inhibitors
Hormone antagonists and related agents				
Tamoxifen (TAM)	371.51	8.69	5.13	Anti-estrogens
4-Hydroxy-N-desmethyl-tamoxifen or Endoxifen (OH-D-TAM)[a]	373.49	9.38 (acidic) 9.34 (basic)	4.94	Anti-estrogens
(Z)-4-Hydroxytamoxifen (OH-TAM)[a]	387.51	10.35 (acidic) 8.70 (basic)	4.93	Anti-estrogens

[a] Active metabolite.
[b] Values obtained from SciFinder Scholar Database, http://www.cas.org/products/sfacad/.

25–30 min, 5% B. The flow rate and the injection volume were set at 0.2 mL min^{-1} and 10 μL, respectively.

The mass spectrometer was operated using both positive and negative ESI modes under the following specific conditions: capillary voltage 3.0 kV, extractor voltage 3 V, source temperature 150 °C, desolvation temperature 350 °C, cone gas flow 50 L h^{-1} and desolvation gas flow 600 L h^{-1}. Nitrogen (> 99.98%) was employed as cone and desolvation gas.

Detection was accomplished in the selected reaction monitoring (SRM) mode using argon (> 99.999%) as collision-induced dissociation (CID) gas at a pressure of 4×10^{-3} mbar in the collision cell. Selected ionization polarities and optimized MS/MS ion transitions for each compound are detailed in Table 2.

3. Results and discussion

3.1. Chromatographic analysis

In general, the use of tandem mass spectrometry detection reduces the need for baseline resolution of all analytes, because it is very rare to find molecules with the same retention time sharing the same unique MS/MS transitions. However, in many instruments the sensitivity of the MS detector decreases as the number of transitions recorded increases, and therefore a certain degree of separation is necessary in order to enable programming of the various SRM transitions into different time windows along the chromatogram.

Table 2
LC retention time (t_R) and selected MS/MS detection conditions for determination of the target cytostatics.

Compound	t_R (min)	Seg.[a]	Ionization	Parent ion	MS/MS transition[b]	Cone (V)	CE (eV)[c]
5-FU	3.25	1	ESI−	$[M-H]^-$	**129.0 > 42.0**	30	15
					129.0 > 86.0	30	15
Car-Pt	3.25	2	ESI+	$[M+H]^+$	**372.0 > 355.0**	25	10
					372.0 > 294.0	25	20
GEM	3.82	3	ESI+	$[M+H]^+$	**264.0 > 112.0**	30	15
					264.0 > 95.0	30	45
Oxa-Pt	4.03	3	ESI+	[M+H] +	**398.0 > 96.0**	40	25
					398.0 > 308.0	40	20
MTIC	4.05	3	ESI+	$[M+H]^+$	**169.0 > 109.0**	10	5
					169.0 > 124.0	10	5
TMZ	4.69	4	ESI+	$[M+H]^+$	**195.0 > 138.0**	25	10
					195.0 > 82.0	25	20
MET	6.49	5	ESI+	$[M+H]^+$	**455.0 > 308.0**	20	20
					455.0 > 175.0	20	35
OH-MET	8.00	6	ESI+	$[M+H]^+$	**471.2 > 324.2**	20	10
					471.2 > 191.0	20	20
VCN	8.71	7	ESI+	$[M+H]^+$	**825.6 > 138.0**	50	40
					825.6 > 807.0	50	45
VBL	9.13	8	ESI+	$[M+H]^+$	**811.6 > 751.0**	50	45
					811.6 > 224.0	50	45
VRB	9.54	8	ESI+	$[M+H]^+$	**779.6 > 658.0**	40	25
					779.6 > 323.1	40	25
IMA	9.71	8	ESI+	$[M+H]^+$	**494.3 > 394.0**	45	35
					494.3 > 99.0	45	35
IF	9.95	8	ESI+	$[M+H]^+$	**261.1 > 183.0**	35	20
					261.1 > 154.0	35	20
CP	10.44	8	ESI+	$[M+H]^+$	**261.1 > 140.0**	30	25
					261.1 > 106.0	30	20
ERL	10.53	8	ESI+	$[M+H]^+$	**394.2 > 278.0**	35	35
					394.2 > 336.1	35	25
ETP	10.60	8	ESI+	$[M+H]^+$	**589.0 > 229.0**	55	15
					589.0 > 185.0	55	40
DOX	11.39	9	ESI+	$[M+H]^+$	**544.3 > 397.0**	25	10
					544.3 > 130.0	25	15
CAP	11.93	9	ESI+	$[M+H]^+$	**360.2 > 244.1**	25	10
					360.2 > 174.0	25	20
OH-D-TAM	12.41	9	ESI+	$[M+H]^+$	**374.3 > 223.0**	35	15
					374.3 > 58.0	35	25
OH-TAM	12.43	9	ESI+	$[M+H]^+$	**388.3 > 72.0**	40	30
					388.3 > 45.0	40	35
TAM	13.74	10	ESI+	$[M+H]^+$	**372.3 > 72.0**	45	25
					372.3 > 45.0	45	30
OH-PAC	13.93	10	ESI+	$[M+H]^+$	**871.0 > 286.0**	20	10
					871.0 > 526.0	20	25
PAC	14.26	10	ESI+	$[M+H]^+$	**854.5 > 105.0**	20	40
					854.5 > 286.0	20	15
CHL	15.34	11	ESI+	$[M+H]^+$	**304.1 > 192.0**	35	25
					304.1 > 168.0	35	35

[a] Segment.
[b] Values in bold correspond to the transitions used for quantification.
[c] Collision energy.

Taking into account the different polarities of the studied cytostatics, a reversed-phase (RP) C18 column was evaluated in an attempt to achieve a suitable separation within the same chromatographic run.

Different modifiers were tested with the purpose of improving not only the response but also the peak shape of the compounds. When ammonium acetate (5 mM) was used, the competitive formation of the corresponding ammonium adducts in the ESI positive mode was observed for most of compounds. This finding was in agreement with the results obtained by Martin et al. [14] and Tuerk et al. [24] for the analysis of ETP and PAC. As most of the studied compounds are better ionized in the positive mode (Table 2), the use of this salt as mobile phase modifier was discarded in an attempt to maximize the ionization efficiency of the protonated molecular ions $[M+H]^+$.

The addition of 0.1% formic acid to the mobile phase improved (approximately 40–60%) the responses of most compounds, especially MET, PAC and the metabolites OH-MET and OH-PAC, which are hardly observed without acid. This is consistent with the fact that acidic conditions generally increase positive-ion ESI response (i.e., low pH conditions favour the formation of the protonated compounds). Nevertheless, the presence of this acid produced a reduction in the negative ion ESI response of 5-Fu by about 25%. On the other hand, the positive ion responses of IMA and VRB slightly decreased with respect to those observed in the absence of modifier, but the peak shape was considerably improved. Hence, acidification of the mobile phase with 0.1% formic acid was finally selected as optimum.

3.2. Optimization of MS/MS detection conditions

The selection of the specific SRM conditions to achieve the maximum sensitivity and selectivity was performed by injection of individual standard solutions of the compounds. Table 2 summarizes the retention times, the most intense SRM transitions, the ionization mode (positive or negative), and the cone voltages and collision energies (CE) selected for monitoring of the various target species.

Positive ionization proved to be the most sensitive ionization mode for all the studied cytostatics (yielding the corresponding protonated parent ion $[M+H]^+$) but 5-Fu. This compound could only be ionized in the ESI negative mode, showing the single negatively charged parent ion $[M-H]^-$.

Two SRM transitions were selected for each cytostatic (Table 2). The most intense transition was used for quantification, while the other one was employed for identification.

3.3. Fragmentation study

The use of LC–MS/MS for cytostatic analysis is growing. However, structural elucidation of the product ions selected for their SRM determination is usually missing. In this work, the fragmentation pattern of each cytostatic considered was investigated. Fig. S1 shows the corresponding product–ion mass spectra obtained for all studied compounds, with the chemical structures proposed for each of the resulting fragments. The strong differences in the chemical structure between the different classes of anticancer drugs cause also great differences in their fragmentation. In general the cytostatics belonging to the same group follow a similar mechanism of fragmentation.

For the alkylating agents CP and IF, the most important fragmentation reaction under the selected conditions was the cleavage of the N–P bond leading to the formation of the ions *m/z* 140 and 120 for CP, and 183, 155 and 92 for IF, as it has been previously reported [12]. The main fragments of CHL are at *m/z* 192, due to the loss of two molecules of CH_2Cl, and at *m/z* 168, due to the loss of CH_2CH_2Cl and $C_3H_5O_2$. For MTIC the most intense ions are at *m/z* 109 and 126; and for TMZ at *m/z* 138 and 82.

MET and its metabolite OH-MET form fragments at *m/z* 308 and 324, respectively, due to the loss of *m/z* 148 ($C_5H_{10}NO_4$). The pyrimidine analogue 5-Fu breaks its ring yielding fragments at *m/z* 86 ($C_2H_2N_2O_2^-$) and *m/z* 42 (CNO^-). The loss of the tetrahydrofuran ring with their substituents of the GEM and CAP molecules gives product ions at *m/z* 112 and 244, respectively.

The group of the vinca alkaloids, VBL, VCN, and VRB, with parent ions of 812, 826, and 780, respectively, breaks in very small fragments. VBL and VRB lose, initially, an acetyl group and give fragments at *m/z* 751 and 658, respectively. VCN loses a molecule of H_2O (18 uma) and gives the fragment at *m/z* 807. Then, the compounds' rings break forming ions at *m/z* 224, 138 and 323 for VBL, VCN, and VRB, respectively.

ETP forms fragments at *m*/*z* 229 and 185. The first one is due to the loss of the glycoside and the dimethoxyphenol rings and the formation of two double bonds in the central molecule. In addition, the last molecule losses the dioxolane ring and gives the fragment at *m*/*z* 185.

The taxanes PAC and OH-PAC give two main fragments at *m*/*z* 569 and 286 in the case of PAC and at *m*/*z* 525 and 286 in the case of OH-PAC due to the breakdown of the ester into two molecules and the formation of a double bond.

DOX losses the glycoside portion and gives a product ion at *m*/*z* 397, and with a further loss of two water molecules leads to a product ion of *m*/*z* 361. In agreement with a previously published method, the transitions from *m*/*z* 544 to 361 and from *m*/*z* 544 to 397 were used for our SRM analysis [6].

The fragments of Car-Pt and Oxa-Pt are difficult to elucidate, but some structures are proposed (see Fig. S1).

The two protein kinase inhibitors (IMA and ERL) undergo different mechanisms of fragmentation. IMA gives two fragments at *m*/*z* 394 and 99 due to the formation of a double bond between the unshared pair of nitrogen belonging to the piperazine ring with the piperazine ring, and the subsequent breakdown of the molecule. The fragment at *m*/*z* 217 corresponds to the rupture of the bond between N and C of the amide and the formation of an aldehyde group. ERL fragments at *m*/*z* 336 and 278 are due to the loss of one and two substituents C_3H_7O remaining one and two hydroxyl groups, respectively.

The anti-estrogens TAM and their metabolites (OH-TAM and OH-D-TAM) break down in very small fragments. TAM and OH-TAM share the same product ions at *m*/*z* 72 and *m*/*z* 45, which correspond to imines.

3.4. Method performance

Fig. 1 illustrates the analysis of the target compounds, which were separated in 30 min. In order to improve the limits of detection (LOD) and quantification (LOQ), they were grouped into 11 segments according to their elution order. The total dwell time per segment was maintained at 1.2 s. The dependence between peak areas and analytes concentrations was investigated with standards prepared in HPLC water (maximum percentage of DMSO 4%) at 7 different concentrations in the range from 5 to 2000 ng mL^{-1} (injection volume 15 μL). Most compounds gave a linear response in the above range, with determination coefficients (R^2) between 0.9917 and 0.9996, whereas MTIC and CHL showed R^2 values of 0.7481 and 0.9776, respectively, because they were not stable under those conditions (see Table 3). Instrumental LODs, defined for a signal to noise ratio (*S*/*N*) of 3, varied between 0.1 and 10.3 ng mL^{-1}, whereas the LOQs (*S*/*N*=10) were in the range 1.0–34.3 ng mL^{-1}. The repeatability in the responses of the system was evaluated with standards at two different concentrations: 50 and 200 ng mL^{-1}. Relative standard deviations (RSDs, %) for 5 injections made in the same day ranged from 0.3% to 6.1%, if MTIC (RSD=30.2–32.9%) and CHL (RSD=20.6–33.3%) are excluded.

3.5. Stability of cytostatics

The fact that some compounds did not present linearity and had relatively large RSDs induced us to think that they were not stable in aqueous solution. Moreover, low recoveries in the analysis of some cytostatics in water samples have been reported by some authors, which could be also due to stability problems. In this context, one purpose of this work was to study the stability of the 24 target cytostatics and metabolites in aqueous solutions and in organic solvents in order to establish the best conditions for preparation and storage of both calibration solutions and water samples.

3.5.1. Effect of the temperature

The first aspect investigated in this respect was the short-term stability of the compounds in water at different temperatures. For this purpose HPLC-water was spiked with the target compounds at 1 μg mL^{-1} (percentage of DMSO 4%), and was injected in the HPLC system immediately after preparation and every 30 min within the next 24 h. During this period, the solution was maintained protected from light at different constant temperatures: 4, 15 and 25 °C. The selection of these temperatures was based on the following premises: 4 °C is the temperature at which most laboratories store water samples from collection to analysis; 25 °C was selected as room temperature and also because it is a temperature used in multiple laboratory exposure experiments and a temperature that can be reached in Southern European rivers in summer; and 15 °C was selected as an intermediate value between the other two, expected to be close to the annual average water temperature of most European rivers. Table 4 shows the percentage of each compound that remained in solution after 24 h at the three studied temperatures. As it can be seen, at 4 °C most compounds were stable, with relative responses within the range 100 ± 20%. The only compounds that showed clearly lower peak signals after 24 h, as compared to the peak signals at time zero, were MTIC (12%) and CHL (20%). Meanwhile, at higher temperatures a reduction of the initial peak signal with time was evident for about half of the compounds investigated and, as expected, the signal decay increased with temperature. At 25 °C, 12 out of the 24 compounds tested, namely, 5-Fu, GEM, TMZ, VCN, VBL, VRB, IF, CP, ERL, ETP, CAP and OH-PAC, were stable (relative response above 80%) for 24 h; 6 compounds, namely, Car-Pt, IMA, DOX, OH-D-TAM, OH-TAM, and PAC, showed some degradation, with relative responses within the margin 50–80%; and the remaining 6 compounds, namely, Oxa-Pt, MET, MTIC, OH-MET, TAM, CHL, were degraded to a large extent, with relative responses below 50% (in fact, the last three compounds were not detected after 24 h at either 25 or 15 °C).

In the light of these results, the stability of the compounds at 4 °C was studied during a longer period of time by analyzing sample aliquots every day during the first 9 days, and after 1, 2 and 3 months of storage in the dark at this temperature. Fig. 2 shows that after 9 days the amount of 11 of the target compounds, namely, Oxa-Pt, MET, its metabolite OH-MET, VBL, IMA, ERL, DOX, TAM, its metabolite OH-D-TAM, PAC, and its metabolite OH-PAC, decreased substantially (to around 50%), MTIC and CHL disappeared completely already after 3 days, and the remaining compounds (5-Fu, Car-Pt, GEM, TMZ, VCN, VRB, IF, CP, ET, CAP, and OH-TAM) were stable (relative response above 80%). After 1 month, all the compounds that were shown to be stable for 9 days, with the exception of OH-TAM, stayed stable (see Fig. 3), whereas after 3 months only Car-Pt and CAP remain unaltered.

These results are in line with those previously published by other authors. Very recently, Tuerk et al. [24], reported recoveries of about 40% and 70% for PAC and docetaxel (another cytostatic that belongs to the family of the taxanes), respectively, in tissues used to wipe sampling areas in pharmacies, further wetted with 1 mL phosphate buffer, and stored at room temperature for 48 h and at −18 °C for 7 days. They studied also other cytostatics, namely, 5-Fu, GEM, MET, CP, IF, and ETP, but recovery rates were closer to 100%. Sottani et al. [6] studied the stability of CP, IF and DOX in human urine, and found that all of them were stable (concentration measured equal to the initial concentration ± 20%) for at least 8 h at room temperature, and for 15 days at −20 °C. Chen et al. [23] found that 5-Fu and PAC were stable in samples of drug-coeluting stents prepared in 15 mL of phosphate-buffered saline (PBS) (pH 7.4; 0.05 M, 1% sodium dodecyl sulphate) for at least 24 h at room temperature (which is in line with our results), and for 1 month at 4 °C (when in our experiment PAC

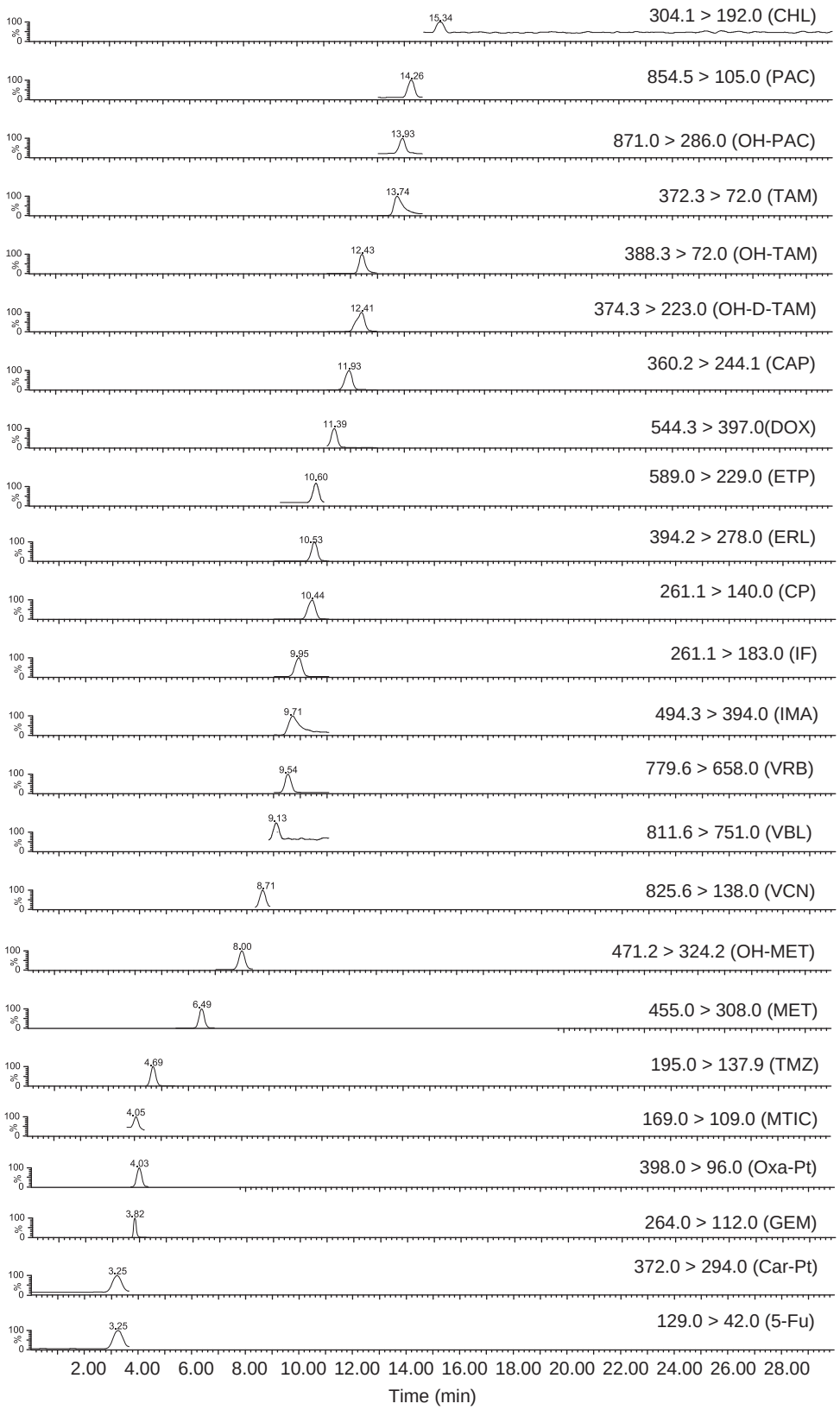

Fig. 1. SRM chromatograms obtained from the analysis of a standard mixture in HPLC-water at 100 ng mL^{-1} by LC–ESI–MS/MS.

decreased in concentration more than 50% after 3 days of storage at the same temperature (4 °C)).

Since about half of the compounds were observed to be unstable at 4 °C after various days, freezing at −20 °C was also evaluated as a possible means to store and preserve both water samples and calibration solutions. For this purpose, samples of HPLC-water spiked with the target compounds at 1 μg mL^{-1} and stored in the dark at −20 °C were analyzed after different storage times (3 days, 9 days, and 1, 2 and 3 months) and compared with a sample prepared in the same way and analyzed immediately after.

Table 3
Determination coefficients (R^2), repeatability (RSD), and limits of detection (LODs) and quantification (LOQ) obtained in the analysis of the target cytostatics by LC–ESI–MS/MS.

Compound	Linearity R^2	RSD (%), $n=5$		LOD (ng mL^{-1})	LOQ (ng mL^{-1})
		50 ng mL^{-1}	200 ng mL^{-1}		
5-Fu	0.9991	5.3	5.0	5.0	16.6
Car-Pt	0.9972	4.7	4.4	2.5	8.3
GEM	0.9982	2.3	2.3	0.6	2.0
Oxa-Pt	0.9978	5.6	1.5	3.0	10.0
MTIC	0.7481	32.9	30.2	7.4	24.7
TMZ	0.9995	3.3	2.5	0.7	2.2
MET	0.9918	1.4	1.6	0.7	2.2
OH-MET	0.9983	3.3	3.1	0.9	2.9
VCN	0.9963	5.6	5.9	10.3	34.3
VBL	0.9924	6.1	1.4	7.5	25.0
VRB	0.9943	4.8	4.5	8.3	27.6
IMA	0.9917	4.8	2.4	8.0	26.7
IF	0.9989	3.7	1.2	2.3	7.7
CP	0.9978	2.0	1.3	0.1	1.7
ERL	0.9940	4.1	3.7	0.1	1.0
ETP	0.9963	4.1	5.0	9.0	30.0
DOX	0.9943	1.3	3.6	0.8	2.5
CAP	0.9996	2.1	1.8	0.6	1.9
OH-D-TAM	0.9975	4.0	2.2	3.0	10.0
OH-TAM	0.9961	1.7	1.8	1.5	5.0
TAM	0.9976	2.8	1.9	5.0	16.7
OH-PAC	0.9955	3.9	4.2	3.0	10.0
PAC	0.9955	4.1	0.3	3.2	10.5
CHL	0.9776	33.3	20.6	9.0	30.0

Table 4
Percentage of compound remaining in solution (HPLC water) after 24 h at three different temperatures (4, 15 and 25 °C), $n=3$.

Compound	4 °C	15 °C	25 °C
5-Fu	101	100	89
Car-Pt	107	85	71
GEM	100	99	103
Oxa-Pt	97	54	26
MTIC	12	0.73	0.87
TMZ	98	91	88
MET	95	46	35
OH-MET	86	–	–
VCN	98	96	93
VBL	91	98	83
VRB	101	105	104
IMA	95	93	77
IF	100	103	94
CP	98	107	103
ERL	96	101	100
ETP	100	104	96
DOX	89	90	77
CAP	97	105	103
OH-D-TAM	88	87	70
OH-TAM	94	91	75
TAM	88	–	–
OH-PAC	92	84	87
PAC	89	72	78
CHL	20	–	–

–: Not detected.

As it can be seen in Fig. 4, freezing improved considerably the stability of various compounds in water. After 3 months of storage at −20 °C, 10 compounds, namely, 5-Fu, Car-Pt, GEM, TMZ, VCN, VRB, IF, CP, ETP, and CAP, remained stable, in contrast to only Car-Pt at 4 °C. Freezing for 1 month would be suitable for the above mentioned 10 compounds plus Oxa-Pt and OH-D-TAM, i.e. for 12 of the 24 target compounds, whereas at 4 °C only 10 compounds were stable for 1 month. Between 1 month and 9 days the differences are minimal: the only compounds unstable for 1 month but stable for 9 days were MET, OH-MET, and OH-TAM; hence, the number of compounds stable at −20 °C for 9 days is 15. Finally, storage at −20 °C for 3 days would be acceptable for all but 7 compounds (MTIC, IMA, ERL, DOX, OH-PAC, PAC, and CHL), which are not stable either under these conditions.

These findings are in agreement with those of Nussbaumer et al. [30], who studied the stability of some cytostatics (cytarabine, GEM, MET, ETP, CP, IF, IRI, DOX, EPI and VCN) on filter papers over 3 months at three storage temperatures (−20, 4 and 25 °C). They found that at 25 °C only IF was stable for 3 months. Concentrations of ETP, DOX, EPI and VCN were already decreased to 20% after 1 week. At 4 °C, the wiping samples were stable for 1 week. After 2 months, the concentrations of DOX, EPI and VCN were inferior to 50% of the initial amount, and at 3 months 50% of ETP was lost. The other compounds were stable for 3 months at 4 °C. At −20 °C all drugs tested were stable for 2 months.

Osawa et al. [31] validated a method for the determination of the contamination of the exterior surface of vials containing platinum anticancer agents (cisplatin and carboplatin) and found that stock solutions of cisplatin and carboplatin prepare in saline solution and ultra-pure water, respectively, were stable at 4 °C for at least 3 months, which is in agreement with our results.

3.5.2. *Effect of the addition of acid*

The effect of adding acid to the water samples as a preservation method was subsequently investigated by using HPLC-water spiked with the analytes (same concentration as above, 1 μg mL^{-1}) and formic acid (0.1%). This solution (pH ca. 2.7) was maintained in the dark at 4 °C and injected every day during 9 days. Fig. 5 shows comparatively the results obtained for the various compounds in the 9th day with and without acid. As it can be seen the behaviour was very variable. Most compounds showed similar results with and without acid. MET and its metabolite OH-MET, compounds that without acid decreased in concentration to about 60 and 40%, respectively, were stable after 9 days when acid was added. MTIC decreased in concentration in both cases, but its degradation in the acidified sample was much less pronounced (60% versus nearly 100% in the non-acidified sample). In contrast, other compounds like IMA, TAM, and its metabolite OH-TAM were even less stable in the presence of acid. Therefore, the addition of acid would only be a solution for MET and its metabolite, not for the rest of compounds.

3.5.3. *Effect of the addition of organic solvent*

Another factor that could improve the stability of sparingly water soluble species is the addition of a miscible organic solvent. This effect was studied with methanol and DMSO. For this purpose standard solutions containing the mixture of the target compounds at 1 μg mL^{-1} were prepared in DMSO:water 4:96, DMSO: water 50:50 and methanol:water 50:50, and were injected daily during 9 days. The peak shape got worse when the percentage of organic solvent increased but the peak areas obtained could still be used to compare and establish the best conditions for preparation and storage of standard solutions and water samples. Fig. 6 shows the results obtained after 3 and 9 days of storage at 4 °C. This figure does not include those compounds that were observed to be stable in 4% DMSO. Moreover, the metabolites OH-TAM, OH-D-TAM, OH-MET and OH-PAC are not represented because they follow the same behaviour as their parent compounds. Hence, in Fig. 6 we can observe the behaviour of Oxa-Pt, MTIC, MET, VBL, IMA, ERL, DOX, TAM, PAC, and CHL. Oxa-Pt, MET, ERL, DOX, TAM, and PAC resulted to be stable in 50% of organic solvent (DMSO and methanol) and no major differences were observed between the third and the ninth days of storage. Meanwhile, the rest of

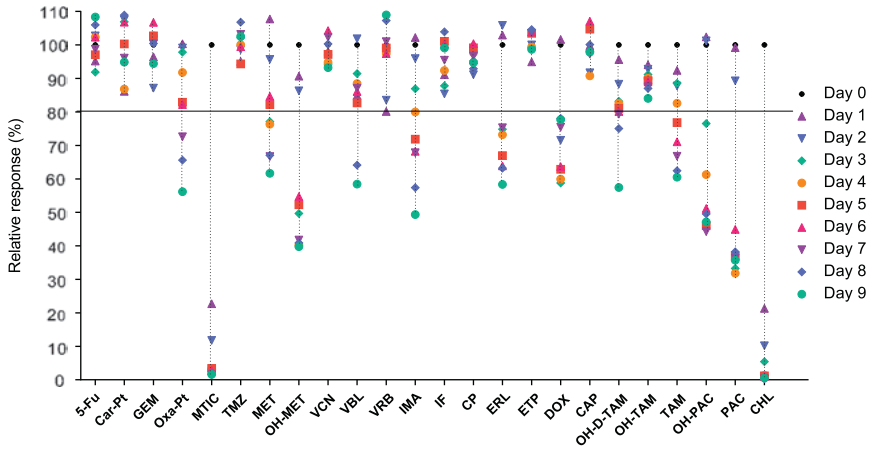

Fig. 2. Stability data of the target cytostatic compounds and metabolites in HPLC water (initial concentration 1 μg mL^{-1}) stored at 4 °C for 9 days.

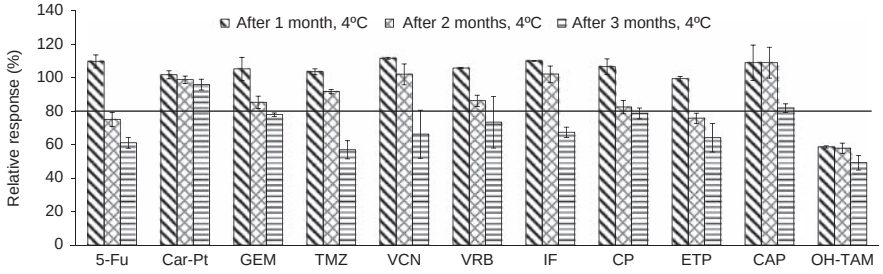

Fig. 3. Relative response of the most stable cytostatic compounds and metabolites in HPLC water (initial concentration 1 μg mL^{-1}) after 1, 2, and 3 months of storage at 4 °C.

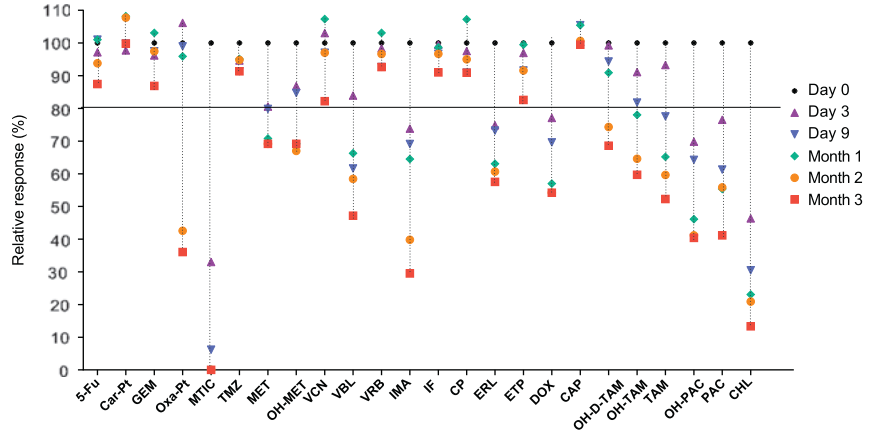

Fig. 4. Stability data of the target cytostatic compounds and metabolites in HPLC water (initial concentration 1 μg mL^{-1}) stored at −20 °C for different time periods up to 3 months.

compounds were degraded to a different extent, and MTIC and CHL were again the least stable compounds. However, the degradation increased when the percentage of organic solvent was lower (4%). Hence, it is clear that the presence of water in the solution plays an important role.

3.5.4. CHL and MTIC

Among all analytes investigated CHL and MTIC stand out clearly as the most unstable compounds.

CHL is a chemotherapy drug that has been mainly used in the treatment of chronic lymphocytic leukaemia. It is a nitrogen mustard alkylating agent and can be given orally. It has also been associated with the development of other forms of cancer [4]. However, to the best of our knowledge, this compound has not been investigated in the aquatic environment and there are no data about its presence or its stability in it. Hence, this compound was studied further in more detail.

When prepared in HPLC-water, this compound was observed to completely disappear after only half an hour at room temperature, and this was accompanied by the appearance of a new chromatographic peak at a lower retention time (6.2 min). The mass spectrum of the newly formed compound obtained in the ESI positive mode exhibited a $[M+H]^+$ ion at *m/z* 269, corresponding to the loss of a chlorine atom from the CHL molecule. Its capillary voltage, collision energy and MS/MS transitions were optimized and the most intense fragment was observed at *m/z* 192, the same as CHL. The second most intense fragment was found at *m/z* 132.

In order to establish whether this product was stable and how much time was necessary for a total conversion, CHL was spiked in

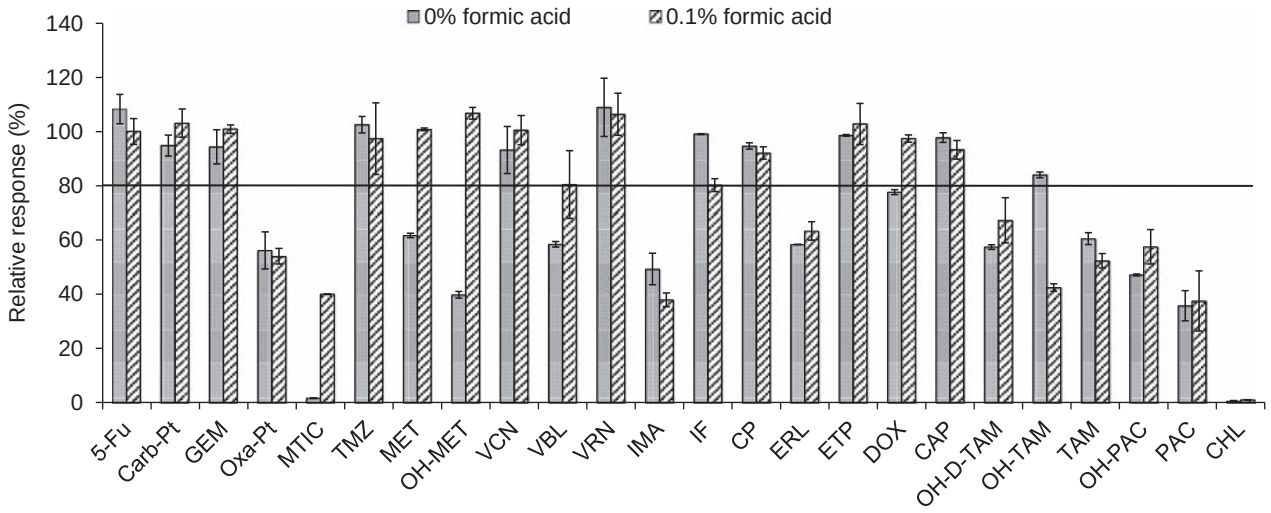

Fig. 5. Effect of the addition of formic acid on the stability of the compounds in water after storage for 9 days.

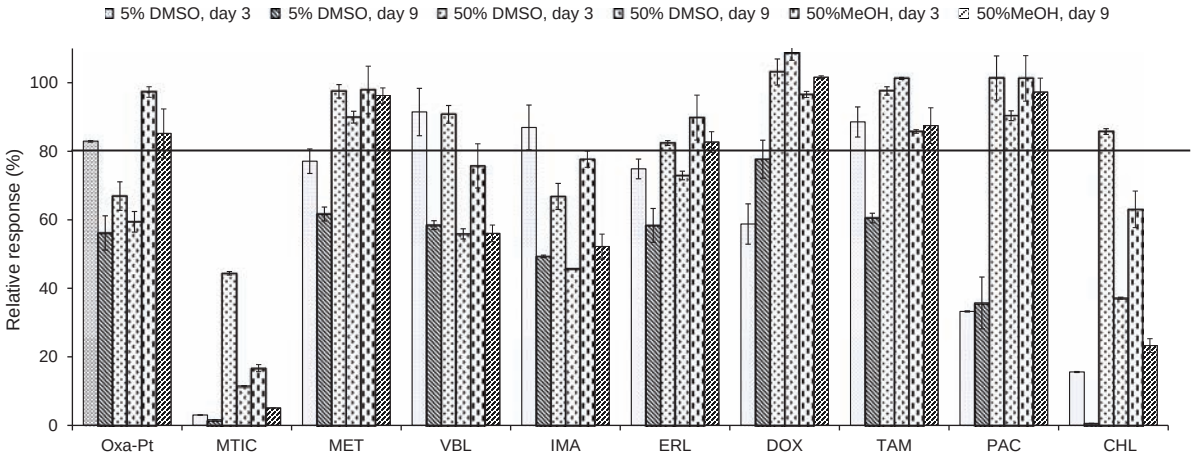

Fig. 6. Effect of the addition of organic solvent on the stability of the compounds in water stored at 4 °C for 3 and 9 days.

HPLC-water at 1 μg mL^{-1} (4% DMSO) and this solution was injected immediately after and at different time intervals during the following 8 days while maintained at different temperatures (4, 15 and 25 °C). Under these conditions, CHL disappeared quickly and the new compound, without a chlorine atom, was formed. Between 15 and 25 °C there were no differences, but at 4 °C the subproduct was formed in a comparatively minor extension.

Fig. S2 (in the Supplementary Information) shows that the response of the subproduct increased with time up until the equilibrium was reached (which at 4 °C occurred after 120 h), remaining stable thereafter for at least 4 more days.

A similar behaviour was observed for MTIC. This compound is a pharmacologically active hydrolysis product of TMZ [28], but our results indicate that MTIC is less stable than TMZ. Fig. S3 (Supplementary Information) shows how MTIC is quickly degraded, remaining only 1% of the compound after 3 and 4 h at 25 and 15 °C, respectively. The degradation at 4 °C is slower than at the other temperatures studied and after 3 h 30% of the compound remained in solution.

4. Conclusions

An LC–MS/MS method has been developed for the analytical determination of 19 cytotoxic drugs and 5 transformation metabolites at the low ng mL^{-1} level. Their stability in water has been studied under different conditions (in relation with temperature, addition of acid, and addition of organic solvent) over 3 months, constituting for many of them the first study of these characteristics carried out. All compounds with the exception of Car-Pt have been shown to be degraded to a higher or lower extent when stored in aqueous solution for a certain time, which may vary from hours to months depending on the temperature (see Table 5). Hence, stock standard solutions shall be prepared in pure organic solvent and the aqueous calibration solutions shall be made up immediately before use. Since some compounds are not readily soluble in methanol (e.g. Oxa-Pt and GEM) and some may also undergo hydrolysis and transesterification in this solvent (e.g. PAC) [32], DMSO appears as a suitable solvent for preparation of stock standard solutions. DMSO presents a high capacity to dissolve substances without interacting with them and is miscible with water. Nevertheless, since DMSO has a relatively high freezing point (18.5 °C) and hence it is solid at, or just below, room temperature, precaution should be taken at not injecting a pure 100% DMSO standard solution directly into the HPLC system.

On the other hand, the fact that many compounds are unstable in water may be behind the lack of studies on cytostatics in water. The inherent instability associated with some compounds makes their quantification extremely difficult in aqueous matrices and this may explain the absence of published data on validated

Table 5
Stability of the compounds in HPLC water (with 4% DMSO) at different temperatures.

Compound	Temperature of storage			
	25 °C	15 °C	4 °C	−20 °C
5-FU	At least 1 day	At least 1 day	1 month	3 months
Car-Pt	< 1 day	At least 1 day	3 months	3 months
GEM	At least 1 day	At least 1 day	2 months	3 months
Oxa-Pt	< 1 day	< 1 day	6 days	1 month
MTIC	< 1 day	< 1 day	< 1 day	< 1 day
TMZ	At least 1 day	At least 1 day	2 months	3 months
MET	< 1 day	< 1 day	6 days	1 month
OH-MET	< 1 day	< 1 day	2 days	1 month
VCN	At least 1 day	At least 1 day	2 months	2 months
VBL	At least 1 day	At least 1 day	7 days	1 month
VRB	At least 1 day	At least 1 day	2 months	3 months
IMA	< 1 day	At least 1 day	3 days	< 1 week
IF	At least 1 day	At least 1 day	2 months	3 months
CP	At least 1 day	At least 1 day	3 months	3 months
ERL	At least 1 day	At least 1 day	3 days	< 1 week
ETP	At least 1 day	At least 1 day	3 months	2 months
DOX	< 1 day	At least 1 day	3 days	< 1 week
CAP	At least 1 day	At least 1 day	3 months	3 months
OH-D-TAM	< 1 day	At least 1 day	7 days	1 month
OH-TAM	< 1 day	At least 1 day	9 days	1 month
TAM	< 1 day	At least 1 day	5 days	1 month
OH-PAC	At least 1 day	At least 1 day	3 days	< 1 week
PAC	< 1 day	< 1 day	3 days	< 1 week
CHL	< 1 day	< 1 day	< 1 day	< 1 day

quantitative analytical methods for the determination of many of them. Further studies should investigate the possible transformation of these compounds into other products, their potential environmental effects, and the eventual risks to humans from environmental exposure to these substances. To this end, the application of the LC–MS/MS method developed to the analysis of environmental samples may be of help, though a previous stage of pre-concentration is necessary.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.070.

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