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An inductively coupled plasma method for determination of cyclophosphamide loaded to polymeric systems ☆

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Abstract

A new method for the determination of cyclophosphamide content of polyalkylcyanoacrylate nanoparticles was developed. The analyses were carried out by inductively coupled plasma atomic emission spectrometry (ICP–AES) by measuring the phosphorus content in the drug. The results obtained by this non-selective technique were compared with those given by high performance liquid chromatography (HPLC) a selective procedure that permits the detection of the cyclophosphamide molecule, and its degradation products. Sensitivity and reproducibility of both procedures were also determined. The ICP–AES method was demonstrated to be valid for sensitivity, precision, accuracy and specificity. In spite of ICP method is not a suitable procedure to analyze the degradation products of cyclophosphamide, the sensitivity of ICP is higher than chromatographic technique. Nevertheless, both procedures are appropriate for the determination of cyclophosphamide-loaded nanoparticles.

Keywords: Cyclophosphamide nanoparticles analysis; Polymeric systems; ICP–AES analysis; Method validation

1. Introduction

Different strategies to develop a more effective and less aggressive citotoxic drugs have been made in recent years. In chemotherapy to obtain a given therapeutic response the correct amount of active drug must be absorbed and transported to the site of action at the right time. The subsequent rate of input adjusted to maintain an active blood level as long as necessary, avoiding, as much as possible, toxic side effects. Of great promise is the use of colloidal delivery systems such as biodegradable nanoparticles [1].

Cyclophosphamide (Fig. 1) is a potent cytotoxic alkylating agent related to nitrogen mustards and valuable in the palliative therapy of certain malignant neoplasms [2]. Association of cyclophosphamide to polymeric systems, such as nanoparticles is an attempt to deliver the cytostatic agent specifically to the tumour in order to reduce side effects of this drug [3]. The evaluation of drug content in nanoparticles is especially com-

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plicated, since it involves separating free from incorporated drug, which is made difficult by the colloidal nature of the polymer matrix.

Different analytical procedures have been described for the determination of cyclophosphamide in biological fluids (plasma, urine) as well as in solid tissues by using non-selective methods, which detect phosphorus present in the drug, and selective methods, such as HPLC, which are capable to detect the intact drug [4].

This study was undertaken in order to develop a new method based on ICP–AES available for studying cyclophosphamide loaded nanoparticles by measuring the phosphorus content in the drug. This technique has been widely applied to the determination of elements owing to its potential advantages of low detection limits, good precision an a wide dynamic ranges of calibration. The results obtained by this technique were compared with those given by a selective method: HPLC with UV-detection. To demonstrate that these techniques are appropriate for the sample matrix, both methods were validated by determining the sensitivity, precision, accuracy and specificity.

2. Experimental

2.1. Materials

Isobutyl-2-cyanoacrylate (IBCA) was purchased from Sigma. Cyclophosphamide and Poloxamer 188 (Surfoxid® F7068) was supplied Prodesfarma (Spain) and Tenneco (Spain), respectively. Dextran 70 (70 SRD) from Pharmacia Fine Chemicals and acetonitrile HPLC grade from SDS S.A. (Spain) were also used. All solvents were of analytical reagent grade. Double-distilled water was used after filtration in a Millipore® system and second vacuum filtration in a helium atmosphere.

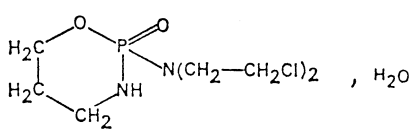


Fig. 1. Chemical structure of cyclophosphamide.

2.2. Preparation of nanoparticles

Polyalkylcyanoacrylate (PACA) nanoparticles were prepared as described by Couvreur et al. [5] using an emulsion polymerization procedure [6]. For the formulation of cyclophosphamide-loaded nanoparticles, the drug (3.5 mg/ml) was added to the polymerization medium (HCl 10^{-3} M containing 0.5% dextran 70, as suspending agent and Surfoxid® F7068: 0.07%, as surface active agents) before dropwise addition of the monomer (IBCA:10 μl/ml). The mixture was stirred for 3 h at room temperature.

2.3. Particle size analysis

Morphometrical properties (average particle size and polydispersity) of nanoparticles were determined by Photon Correlation Spectroscopy in a Malvern Autosizer II C (Malvern Instruments, Malvern, U.K.) [7]. Samples were diluted before measurement with twice-distilled water freshly filtered through a membrane filter (Millipore®).

2.4. Entrapment of cyclophosphamide into nanoparticles

The amount of cyclophosphamide entrapped into nanoparticles was estimated by two procedures: atomic emission spectrophotometry, using an ICP and HPLC with UV-detection.

Determination of cyclophosphamide linked to the nanoparticles was carried out by separating free drug from cyclophosphamide-loaded nanoparticles by ultracentrifugation of the suspension at 40,000 rpm, for 2 h at 20°C, in a Kontron ultracentrifuge. Phosphorus or cyclophosphamide contents were measured (by ICP and HPLC respectively) in both supernatant (free drug) and sediment (previous NaOH hydrolysis, in HPLC method). Drug entrapped (sediment) was expressed as the percentage of drug initially

Table 1
Morphometrical properties (size average Z, polydispersity Q) and association efficiency (AE) of cyclophosphamide nanoparticles

Cyclophosphamide–PACA polymerization medium composition	Z (nm)	Q	AE (%)	
			HPLC	ICP
Dextran 0.5% Surfoxid® F7068	217.6	0.074	21.79	22.58

dissolved in the polymerization medium, linked to the carrier.

2.5. HPLC measurements

HPLC determinations were carried out in a liquid chromatograph with a constant-flow-rate pump and diode array detector (model HP1090, Hewlett Packard, U.S.A.), integrator (model HP-3396D, Hewlett Packard) and Hewlett Packard ink jet printer. Samples were chromatographed using an analytical ODS–Hypersyl column (Hewlett Packard of 10 cm length, 4.6 mm internal diameter and 5 μm particle size). The mobile phase had a flow rate of 1.5 ml/min under isocratic conditions of acetonitrile–water (30:70, v/v) [8]. The UV detector was set at 195 nm. Each sample was injected in triplicate and the results were averaged to obtain the value of the concentration. All samples were chromatographed in order to assess the reproducibility and linearity of the method. Linearity of the liquid chromatography assay was determined by linear regression; peak areas for the antineoplastic agent were plotted on a linear scale vs. drug concentration. Reproducibility of assays was also determined.

2.6. ICP measurements

A Jobin Yvon instrument, was used to determine the amounts of phosphorus in cyclophosphamide by inductively coupled plasma (ICP) at 178,287 nm with a Meinhard system, TR 30-C3, as a nebulizer. After ultracentrifugation (40,000 rpm, for 2 h, at 20°C), amounts of phosphorus were measured in both sediment (linked cyclophosphamide) and supernatant (free cyclophosphamide). Concentration of phosphorus in nanoparticles suspension was also measured. Sediment and nanoparticle suspension were diluted (1:5) before ICP analysis, in order to avoid the effect of polymeric matrix in the plasma torch.

3. Results and discussion

3.1. Assay development

Morphometrical properties of cyclophosphamide nanoparticles (size average, polydispersity: Q) containing the same percentage, 0.5% of dextran 70 and 0.2% of Surfoxid® F68, are shown in Table 1, in which the average amount of cyclophosphamide entrapped (%) obtained by both experimental procedures used (ICP and HPLC) is also shown.

3.2. Method validation

The ICP analytical assay method of cyclophosphamide loaded nanoparticles was validated to demonstrate the specificity, sensitivity, linearity precision and accuracy of the method, comparing with results obtained by HPLC classical procedure.

3.2.1. Selectivity

The emission spectra of cyclophosphamide, phosphate solutions or primary phosphorus standard were performed at the phosphorus emission wavelength 178.287 nm (Fig. 2a). A high performance liquid chromatogram of cyclophosphamide aqueous acid solution (corresponding of supernantant fraction after centrifugation of final colloidal system) containing stabilizers appears in Fig. 2b. The drug was eluted in a single peak with a retention time of $1.45 \pm 5\%$ min.

In order to apply the ICP method proposed to the analysis of cyclophosphamide loaded

nanoparticles, spectral and background interferences of nanoparticle matrix were investigated. In this way, the influence of commonly used stabilizers, excipients and additives used in the nanoparticles manufacturing, was studied by preparing aqueous acid solutions containing 35 ppm of drug and different amounts of the foreing compounds.

As nanoparticles were usually performed in presence of stabilizers (0,5% dextran 70 and 0,2% Surfoxid® F7068 or sodium laurylsulphate) and also a little percentage of this polysaccharide was entrapped into the colloidal system, we added dextran 70 and a surface active agent to the cyclophosphamide standard solutions in order to check the influence of this polymer on HPLC or ICP assays. Additional peaks were obtained for dextran and surface active agents (Fig. 2b). Differences in retention time of cyclophophamide and technique signal (areas or emission intensity) between different solvents used (HCl or HCl with stabilizers) were not significant. No interferences were found in presence of nitrogen, sulphur or sodium as can be seen in Fig. 3. Other compounds as glucose, manitol, cellulose, starch, propylenglycol and dextran neither acts as interference.

As the ICP method described is a non-selective method which detects the cyclophosphamide content as a phosphorus present in the drug, phosphate solutions or another phosphorus compounds could be avoid. In this way, ICP is not a suitable procedure for studies of metabolism of cyclophosphamide because the majority of metabolites of this drug [9] containing phosphorus compounds.

Hence, the proposed method may be considered at sufficiently selective for the analysis of the association efficiency of cyclophosphamide in nanoparticles.

3.2.2. Linearity

Cyclophosphamide showed a linear detector response ($r > 0.998$) in the concentration range of 0.35–4.90 mg/ml, and presented a good standard deviation. All values are listed in Table 2. *F*-test was applied in order to determine some differences between two linear regression with or without constant term ($y = bx + a$, or $y = cx$, respectively). *F*-value [10] obtained ($P = 0.05$) did not show significant differences between two procedures which indicate that systematic mistakes are not present in analytical method. In the ICP procedure a good linear relationship between con-

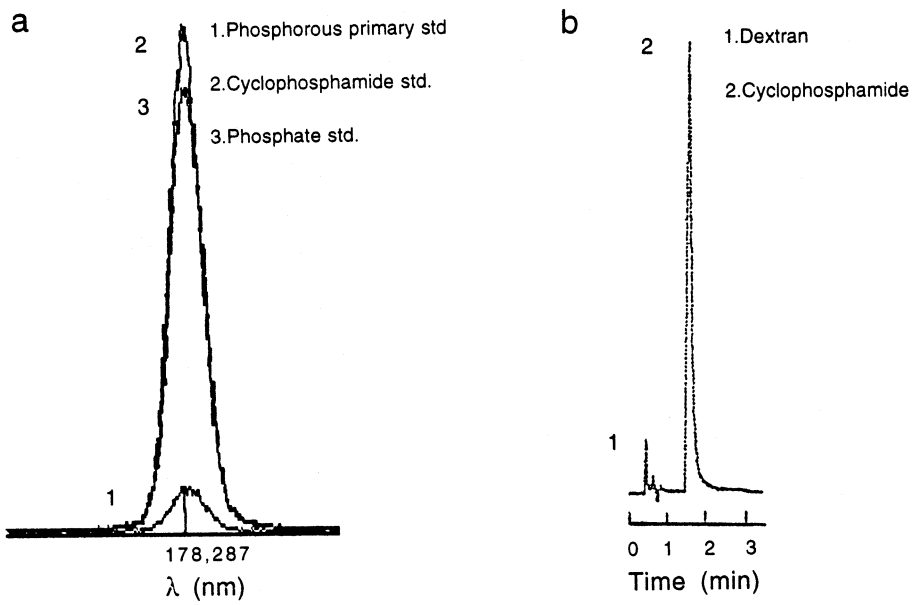


Fig. 2. Signal detector reponse in ICP (a) or HPLC method (b).

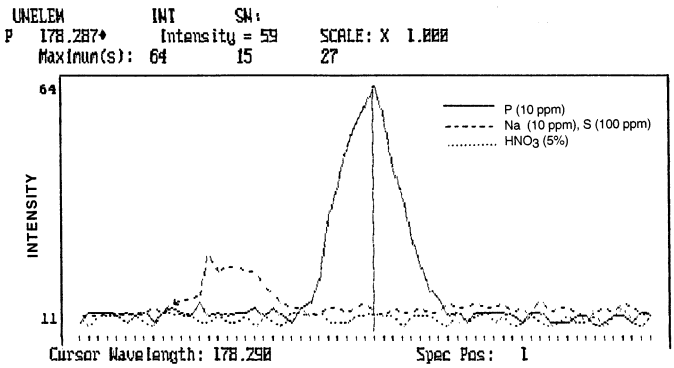


Fig. 3. ICP Interferences analysis.

centration and atomic emission response of cyclophosphamide was observed ($r > 0.999$) at the same concentration range analyzed (Table 3). *F*-value obtained does not show significant differences between linear regression with or without constant term. Significant variations in linearity in water solutions of cyclophosphamide containing dextran and surface active agents, were not found in both analytical procedures (Fig. 4).

3.2.3. Limits of detection and quantification

The limit of quantification, defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision and variability was calculated in ICP method from the signal of 30 samples of blank by means of Eq. (1)

$$\mathrm{LOQ} = \frac{\mathrm{LOD} - \bar{S}_{\mathrm{bl}}}{m} \quad (1)$$

where LOD is the limit of detection, calculated through $\mathrm{LOD} = \bar{S}_{\mathrm{bl}} + 3\mathrm{SD}_{\mathrm{bl}}$, where $\bar{S}_{\mathrm{bl}}$ is the mean blank signal, $\mathrm{SD}_{\mathrm{bl}}$, standard deviation of the blank measures and m the slope of calibration curve ($y = 0.002x + 0.004$, $r > 0.99$) [11]. The value obtained for LOD and LOQ were 0.011and 0.51 ppm, respectively.

In HPLC where it is not possible to carry out blank determinations, an approximate estimation of statistic detection limit (LOD), could be calculated through Eq. (2)

$$\mathrm{LOD} = \frac{3\mathrm{RSD}}{b}\sqrt{\frac{n-2}{n-1}} \quad (2)$$

where RSD is the regression standard deviation of the instrument signal. The limit of detection of the HPLC method calculated was 0.17 mg/ml being the limit of quantification slightly higher, in accordance with results obtained by others authors [12]. The high values obtained for the detection limit could be due to the fact that measurements has been performed at wavelength of 195 nm in which the absortivity of drug is lower that obtained at 190 nm [13].

3.2.4. Precision and accuracy

The inter-day reproducibility (confidence level 95%) of the HPLC assay was determined by injecting aliquots ($n = 6$) of cyclophosphamide nominal solution (3.5 mg/ml). At the same time, these samples were also evaluated by ICP (Table 4). For a nominal solution of 3.5 mg/ml, the variation coefficient shown by HPLC was 1.253%, whereas that for ICP was 0.112%, which indicates that ICP gives better reproducibility. The intra-day reproducibility (ISO 5725) (confidence level 95%) was assessed by ICP of three analysis repetitions of samples ($n = 6$) at a concentration of 3.5 mg/ml carried out during 3 days. At the same way, these samples were also evaluated by HPLC. For a solution of 3.5 mg/ml, the standard deviation shown by ICP was 5.7×10^{-3}, whereas that for HPLC was 4.89×10^{-2}. Although the precision and accuracy are suitable for both procedures, results obtained by ICP in the concentration range analyzed show a better precision and accuracy.

Table 2
Peak areas obtained for different concentrations of cyclophosphamide, by the HPLC method[a]

C (mg/ml)	Area 1	Area 2	Area 3	Area average
0.350	917,400	918,404	917,475	917,760
0.700	1,696,489	1,708,337	1,692,816	1,699,214
1.400	2,545,920	2,535,177	2,523,641	2,534,913
2.100	3,882,572	3,846,034	3,892,827	3,873,811
2.800	5,030,334	5,066,282	5,030,926	5,042,514
3.500	6,347,038	6,348,410	6,349,581	6,348,343
4.200	7,794,113	7,808,735	7,745,467	7,782,772
4.900	9,258,332	9,272,968	9,279,607	9,270,302

[a] $y = bx + a$, $b = 1{,}802{,}903.4$, SD(b) = 45,461.6, $a = 187713.3$, SD(a) = 133,244.4, $r = 0.9981$; $y = cx$, $c = 1{,}857{,}396.3$, SD(c) = 27,555.4, r = 0.9975; F, $F_{exp} = 1.985$, Significance for $P = 0.05$, $F > 5.59$.

3.3. Stability of the analysis

Earlier studies on the cyclophosphamide solutions stability had demonstrated that these drug is not very stable at room temperature [13]. The loss of drug from water solutions after storage for 8 h at room temperature, assayed by HPLC, was 1.5%, being this behaviour independent of pH between pH values of 2–10 [14]. Therefore, samples must be analyzed by HPLC on the same day or should be stored at lower temperature [13]. In ICP method proposed, stability of samples is not a relevant factor because this method is not specific for the analysis of cyclophosphamide molecule and the content of phosphorus in the solutions remains invariable with time.

3.4. Application of ICP method to analysis of cyclophosphamide-loaded nanoparticles

The sensitivity of ICP, for determination of cyclophosphamide loaded nanoparticles, calculated from $[D > 1.96\ (SD/\sqrt{n})]$was $D > 0.001$ (for $n = 18$), better than HPLC ($D > 0.56$), at the concentrations interval analyzed. This agrees with results obtained by Dominicy et al. [15] using ICP–AES for the determination of other metals levels in serum and urine.

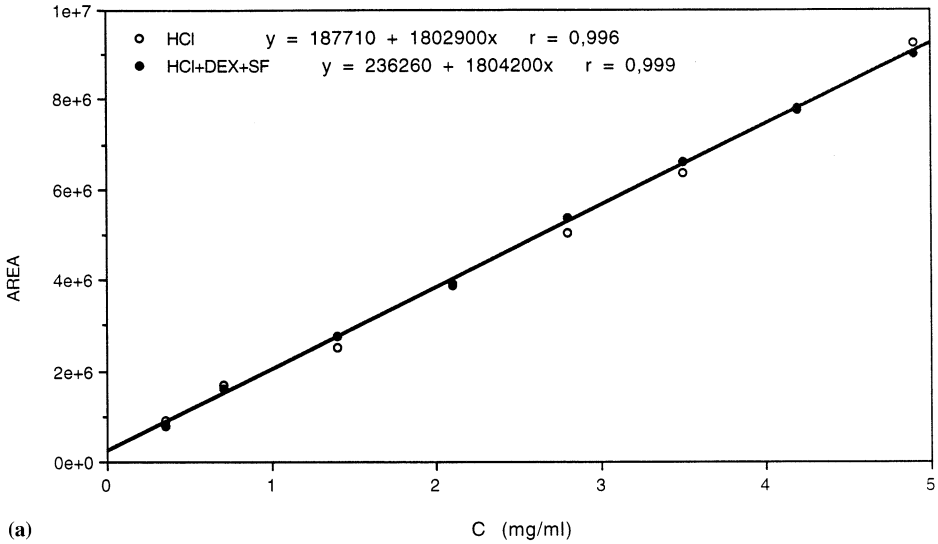
In spite of usually association efficiency of drug in nanoparticles is determined by difference between amount of drug present in the initial solution and drug contained en the supernatant after ultracentrifugation of samples [16,17], better results were obtained when drug content was also evaluated in the nanoparticles (sediment), previous hydrolysis with dimethylformamide or NaOH.

Because cyclophosphamide does not have a strong chromophore, it was necessary to monitor the drug at very low wavelength (190–195 nm)[13]. At these low wavelengths the baseline noise of the UV detector is significant. On the other hand, reagents used for the hydrolysis of nanoparticles usually show an UV-absorption peak that constitutes an interference with the cyclophosphamide peak and so it is not possible the determination of drug content in the sediment by HPLC. On the contrary, the ICP method

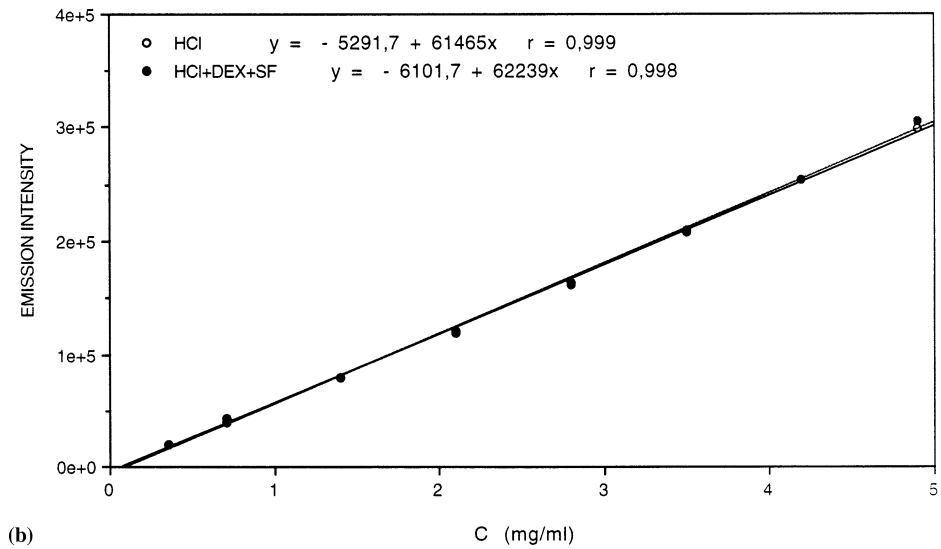
Table 3
Emission intensity obtained for different concentrations of cyclophos phamide, by the ICP method[a]

C (mg/ml)	El 1	El 2	El 3	El average
0.000	59	66	71	66
0.350	19210	19244	19035	19163
0.700	39746	39472	39941	39720
1.400	79742	79860	79975	79742
2.100	119290	120760	121860	120637
2.800	162590	160055	161615	162753
3.500	206470	209860	209225	208518
4.200	253465	256900	252500	254288
4.900	301095	300605	295490	299063

[a] $y = bx + a$, $b = 60{,}979.0$, SD(b) = 655.3, $a = -3619.0$, SD(a) = 1810.9, $r = 0.9996$; $y = cx$, $c = 59{,}928.4$, SD(c) = 490.4, r = 0.9994; F, $F_{exp} = 3.994$, Significance for $P = 0.05$, $F > 5.12$.



(a)



(b)

Fig. 4. Effect of solvent solution composition in the signal technique (area or emission intensity).

allows evaluation of the drug content in the nanoparticles (sediment) and so improved the accuracy determination of cyclophosphamide entrapped into the nanoparticles.

On the other hand, the ability to monitor the kinetic degradation of cyclophosphamide by HPLC is an important advantage against ICP technique. The ICP method has an additional advantage in the determination of cyclophosphamide against HPLC procedure consisting of in the possibility to use, as standards, solutions of phosphates because this technique detect the phosphorus present in the drug. Results obtained for linear regression using cyclophosphamide standard solutions ($y = 0.002x + 0.004$; $r > 0.999$) or phosphate standard solutions, prepared from a

Table 4
Reproducibility of ICP and HPLC methods

Samples	ICP (El) 3.5 mg/ml	HPLC (Area) 3.5 mg/ml
1	208,940	6,563,486
2	208,440	6,587,694
3	208,336	6,578,997
4	208,546	6,674,532
5	208,187	6,757,237
6	208,564	6,765,580
x	208,502	6,654,587
SD	233.89	83,408.80
VC	0.112	1.253

primary standard of phosphate containing 1000 ppm of phosphorus ($y = 0.002x + 0.009$; $r > 0.999$), not show significant differences at the interval of concentrations assayed (0–1050 ppm). The limit of detection (0.009 ppm) and the limit of quantification (0.64 ppm) were in the same order that obtained using cyclophosphamide standard solutions. This fact is very interesting in order to decrease the manipulation of toxic drugs as cyclophophamide.

4. Conclusions

Analysis of cyclophosphamide as metal, in nanoparticles by a non-specific method, shows similar values to these obtained when a specific procedure for determination of intact drug (HPLC) was used. The ICP–AES method proposed was demonstrated to be valid for sensitivity, precision, accuracy and specificity. In spite of ICP method is not a suitable procedure to analyze the degradation products of cyclophosphamide, the sensitivity of ICP is better than chromatographyc technique. These results indicate that ICP is suitable for cyclophosphamide nanoparticle content analysis and represents an important step in the improvement the manipulation with citotoxic drugs.

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