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Journal of Chromatography A, 1088 (2005) 169–174
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Analytical procedure for the determination of chlortetracycline and 4-epi-chlortetracycline in pig kidneys

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Available online 22 January 2005

Abstract

A liquid chromatography-diode array detection (LC-DAD) procedure has been developed for assaying chlortetracycline (CTC) and its 4-epimer (4-epi-CTC) residues in pig kidneys. The procedure involved extraction with 0.1 M oxalic buffer followed by protein precipitation with trichloroacetic acid. Further solid-phase extraction (SPE) clean-up on a Strata X polymeric cartridge was allowed to obtain an extract suitable for LC analysis. Chromatographic separation was carried out on a C_8 analytical column, using isocratic elution with methanol–acetonitrile–0.01 M oxalic acid (15:15:70, v/v/v) at ambient temperature. The flow-rate was 1.2 ml/min and the eluate was analysed at 365 nm. The whole procedure was evaluated according to the requirements of the European Union regulation 2002/657/EC determining specificity, decision limit (CCα), detection capacity (CCβ), trueness, precision and robustness during validation process. The decision limit (CCα) was 674.8 μg/kg for CTC and 683.6 μg/kg for 4-epi-CTC. The detection capacity (CCβ) was 683.6 and 696.3 μg/kg for CTC and 4-epi-CTC, respectively. The recoveries of CTC and 4-epi-CTC from spiked samples at the levels of 300, 600 and 900 μg/kg (0.5 × MRL, 1 × MRL and 1.5 × MRL) were higher than 70%. This method has higher throughput than reported previously extraction method with oxalic acid and acetonitrile used for dechelation and deproteinization.

Keywords: Chlortetracycline; 4-epi-Chlortetracycline; Residues; Kidneys

1. Introduction

Chlortetracycline (CTC), the first member of the tetracycline family, is a broad-spectrum antibiotic widely used in veterinary practice for the treatment of bacterial infections [1,2]. CTC is active against aerobic and anaerobic Gram-positive and Gram-negative bacteria as well against some microorganisms that are resistant to cell-wall-inhibitor antibacterial agents. CTC is actively transported into the cells of susceptible bacteria and acts by inhibiting protein biosynthesis after binding to the 30S ribosome subparticle. CTC is licensed for the treatment of respiratory and systemic infections in pigs, poultry and other farm animals [3,4].

Because of common use, residual CTC may occur in tissues collected from slaughtered animals if the adequate withdrawal time has not been observed [5,6]. Therefore, edible tissue animal origin may be a potential hazard for the consumers. To protect consumers' health, the European Community (EC) legislation on veterinary drug residue has laid down maximum residue limits (MRL) for CTC, including its 4-epimer (4-epi-CTC) at 600 μg/kg in kidney, at 300 μg/kg in liver (and in skin + fat) and at 100 μg/kg in muscle, for all food-animal producing animals [5,6].

Under mildly acidic (pH 2–6) conditions, CTC reversibly epimerises to 4-epi-chlortetracycline (4-epi-CTC) which is an antibacterially inactive compound (Fig. 1). This requires the development of methods that are capable in separating CTC and 4-epi-CTC in tissues of animal origin.

A number of analytical procedures are currently available for the determination of CTC and 4-epi-CTC residues in animal tissues. A comprehensive review of these methods is included in article on the chromatographic analysis of tetracycline antibiotics in food [7]. Generally, analytical method described in literature show an extraction step with mild acid (pH 2–4) solvents followed by solid-phase extraction (SPE)

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doi:10.1016/j.chroma.2005.01.007

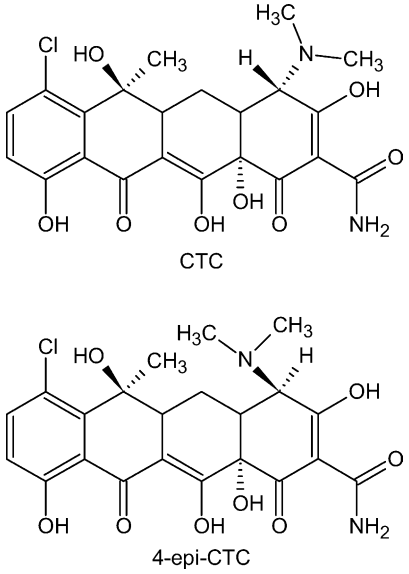

Fig. 1. Structure of CTC and 4-epi-CTC.

or metal chelate affinity chromatography (MCAC) as clean-up procedures [8–21]. However, some these procedures can be laborious or the use of some type disposable cartridges can lead to poor and inconsistent recoveries. The recovery variability problem has also been reported by other authors [10]. It is therefore recommended to use of SPE cartridges and LC analytical columns containing a high purity silica or polymer materials.

The method, routinely used in our laboratory [20], prepared for assaying parent tetracycline agents in animal tissues. However, it was not assaying the suitability to the simultaneous determination of CTC and 4-epi-CTC in pig kidney.

This paper reports improvement of sample preparation approach by using the combination of oxalic buffer and trichloroacetic acid (TCA) solution for extraction step followed by suitable SPE cartridges, resulting in further increases in throughput similar to those achievable by other analytical protocols described for the determination of CTC and its 4-epimer [18,21,22]. The whole procedure was validated according to the requirements of the European Union regulation 2002/657/EC [23].

2. Experimental

2.1. Reagents and materials

Acetonitrile, methanol (HPLC grade), and oxalic acid were purchased from J.T. Baker (Deventer, the Netherlands). Sodium oxalate and trichloroacetic acid were obtained from (P.O.Ch., Gliwice, Poland). Deionised water was purified by a Mili-Qplus system from Millipore (Bedford, MA, USA). Analytical standards of CTC and 4-epi-CTC were supplied by Sigma (Poole, UK). The following SPE disposable cartridges were tested: Bakerbond octadecyl (C_{18}) 500 mg (catalogue No. 7020-03), Bakerbond octyl (C_8) 500 mg (7087-03), Bakerbond styrene-divinylbenzene (SDB1) (7519-02) from J.T. Baker (Devender, the Netherlands). Oasis HLB (Part No. 186000115) from Waters (Milford, MA, USA), and Strata X (8B-S100-ECH) from Phenomenex (Torrance, CA, USA).

2.2. Buffer and standard solutions

A pH 3.5 oxalic buffer (1000 ml) was prepared by combine 500 ml of 0.1 M sodium oxalate with 500 ml of 0.1 M oxalic acid. The solution was filtered through a 0.45 μm PTFE filter from Milipore (Bedford, MA, USA).

Individual stock solutions of CTC and 4-epi-CTC (1 mg/ml) were prepared by dissolving 10 mg of the analytical standards in 10 ml of methanol and stored in the dark at −20 °C (stable for at least 6 months). Combined stock solution (100 μg/ml) was prepared by diluting 1 ml of each individual stock solution with 8 ml of methanol and stored in the dark at 4 °C (stable for at least 3 month). Working standard aqueous solutions of the analytes were prepared by serial dilution of the combined stock solution with oxalic buffer at pH 3.5 and stored in the dark at 4 °C (stable for at least 2 weeks).

2.3. Sample preparation equipment

Centrifugation of the samples was performed in a Verifuge 3.0R a refrigerated centrifuge (Heraus, Germany). A vortex stirrer type IKA (IKA, Labortechnik, Stanfen, Germany) was used to mix samples during treatment. The pH of the buffer solution was adjusted with 780 pH Meter (Metrohm, Switzerland). Evaporation under nitrogen was conducted in a Reacti-Vap evaporator from Pierce (Rockfold, IL, USA). A Polytron, Kinematica (Littau-Luze, Switzerland) was used to homogenisation of the kidney samples with extraction solutions. A SPE 12G vacuum manifold (J.T. Baker, Devender, the Netherlands) was used for solid-phase extraction clean up.

2.4. Blank samples

Kidney samples were obtained from the healthy adult pigs that were not treated with any antibacterial compounds within the previous 4 weeks. The samples were minced, accurately weighed (2.5 g wet mass) and deep-frozen at −20 °C until the time of analysis.

2.5. Spiking procedure

In order to investigate the spiking solutions and time exposition on extraction procedure, the CTC and 4-epi-CTC working standard solutions were prepared in water, oxalic buffer and methanol, respectively. The samples were spiked with working standards at the concentrations corresponding to level of 600 μg/kg and were kept in the dark at ambient temperature. The samples were analysed as follow: (a) directly, (b) 15 min after spiking, and (c) 60 min after spiking.

2.6. Sample extraction

An accurately weighed 2.5 g amount of sample was transferred into 50 ml centrifuge tube. To the sample, 2.5 ml of 0.1 M oxalic buffer was added and stirred with a glass rod for 1 min. Further, 2.5 ml of 30% TCA solution was added, and the tube content was stirred with a glass rod for 1 min. An additional volume of 15 ml of oxalic buffer was added to the tube and the tube content was homogenised for 5 min with speed 3500 rpm. After homogenisation, the tube was transferred to the centrifuge and the sample was centrifuged at 3500 rpm for 15 min (temperature 4 °C). Supernatant was separated from solid phase and filtered.

2.7. Solid-phase extraction

The blank kidney samples were used to obtain raw extracts according to procedure described in Section 2.6. The aqueous sample extracts were spiked with CTC and 4-epi-CTC at the level of 30 μg/ml.

The tested SPE disposable cartridges were preconditioned with 3 ml of methanol followed by 3 ml of water. The spiked sample extracts were passed through the cartridges at a flow rate of approximately 0.5 ml/min. The cartridges were washed with 3 ml of water. After air drying, elution was accomplished using 3 ml of methanol. Alternatively, 5 ml of methanol and 5 ml of mixture of methanol–0.1 M oxalic buffer (95:5, v/v) were used as elution solvents to the test of differences in CTC and 4-epi-CTC extracting capacity. The eluates were evaporated to dryness at 40 °C, under a gentle stream of nitrogen gas. The dry residues were dissolved in 0.5 ml of mobile phase for injection into liquid chromatograph. The tube contents were vortexed and the reconstituted samples were transferred to autosampler vials. The volumes of 50 μl were injected onto LC analytical column.

2.8. Liquid chromatography

Analyses were carried out on a Varian ProStar Series liquid chromatograph (Walnut Creek, CA, USA) equipped with an autosampler, a degasser and a mixer of mobile phase. A photodiode array detector ProStar 330 was used to analyse the tested solutions with wavelength of 365 nm (min. 250 nm, max. 500 nm); sampling intervals, 400 ms; slit width, 2 nm LC control, data acquisition and peak integration was performed by system controller utilizing interface for the communication with the chromatograph workstation.

The chromatographic separation was performed with isocratic elution on a Hypersil C_8 (250 mm × 4.6 mm, 5 μm) analytical column (ThermoQuest, Kleinosteim, Germany) coupled with a RP C_8 guard cartridge (4.0 mm × 3.0 mm, 5 μm). The mobile phase consisted methanol–acetonitrile–0.01 M oxalic acid (15:15:70, v/v/v). Separation of the analytes was accomplished with flow of 1.2 ml/min at ambient temperature.

2.9. Validation study

Since the EU regulation on MRLs is the sum of the CTC and its 4-epimer, validation was carried out for both compounds. The evaluation of the whole procedure for the determination of CTC and 4-epi-CTC residues in pig kidneys was carried out according to the requirements of the European Union Regulation 2002/657/EC [23]. During the validation process, the linearity of the assay, repeatability (precision), recovery (trueness), specificity, decision limit (CCα), detection capability (CCβ), stability and robustness have been evaluated.

To the validation purposes, the blank kidney samples were spiked with the CTC and 4-epi-CTC working solution to levels corresponding 0.5 × MRL, 1 × MRL and 1.5 × MRL, respectively. The spiking solution were mixed with samples using vortex mixer, the samples were kept in the dark at ambient temperature for 15 min before analysis.

3. Results and discussion

3.1. LC separation

CTC and 4-epi-CTC are the well-known compounds forming chelates with divalent and trivalent cations, additionally they also strongly interact with the silanols groups locating in the chromatographic stationary support. Free silanol groups and metal impurities in column materials cause peak broadening and tailing on reversed-phase sorbents, which makes it impossible to achieve chromatographic separations suitable for residue analyse of tetracycline antibiotics [7].

Usually, the chromatographic columns packed with highly purified silica or polymer sorbents are used for the chromatographic separations of CTC and other compounds from the tetracycline family. In order to avoid forming chelates, reversed phase column chromatography uses mobile phases with low pH environment [7,12–17].

As detailed in a previous paper [20], the separation of parent tetracycline agents was able on a PLRP-S analytical column using acetonitrile and 0.01 M oxalic acid (25:75, v/v), as mobile phase. The preliminary studies indicated that the previously developed chromatographic conditions were not suitable for the selective separation of CTC and its 4-epimer.

In this LC method, separation was made possible by using a Hypersil C_8 analytical column and the mobile phase containing acetonitrile, methanol and 0.01 M oxalic acid. At sum of organic modifier concentrations higher than 30%, the peaks were not fully separated and their retention times were very low. The optimal separations were achieved with acetonitrile, methanol and 0.01 M oxalic acid in 15:15:70 (v/v/v) mixture with retention times for 4-epi-CTC between 6.06 and 6.10 min and for CTC between 8.11 and 8.15 min. The typical chromatogram corresponding to a separation under developed conditions is shown in Fig. 2a.

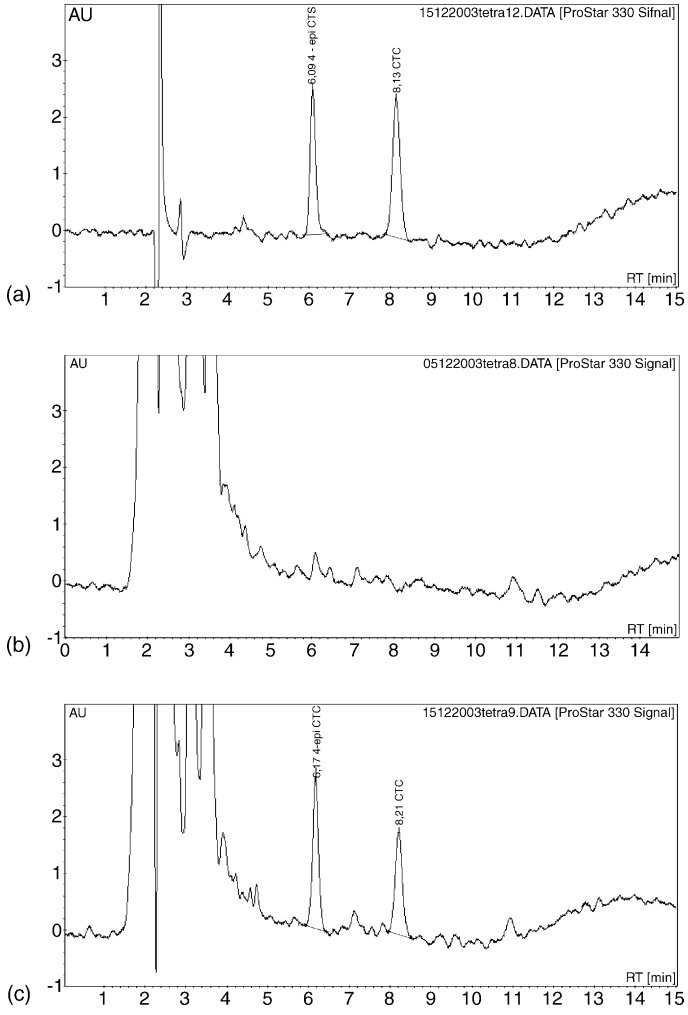

Fig. 2. Chromatogram obtained from LC-DAD analysis of (a) standard solution of CTC and 4-epi-CTC; (b) blank pig kidney sample; (c) pig kidney sample spiked at 300 μg/kg with CTC and 4-epi-CTC.

The most screening chromatographic assays employ UV detection (350–360 nm) for TCs and their epimers [13,20,21,23]. Two absorption maxima (270 and 365 nm) were found for CTC and 4-epi-CTC. At 270 nm, the interferences were observed with endogenous compounds isolated from kidney samples. The use of 365 nm wavelength improved the resolution of the analytes without any interferences from matrix compounds. The identification criteria of decision 2002/657/EC for full scan UV–vis detection were accomplished for all analysed solutions.

3.2. Isolation

Several extraction method have been developed for the isolation of CTC and other tetracycline compounds from biological samples [8–20,22]. However, some of them involve complex extraction and time-consuming steps with great variability in the extraction ratio. As tetracyclines form a chelate complex with metal ions and bind proteins, it has been considered that strong acid, organic and acidic deproteinizing agents are suitable to extract tetracyclines from biological samples [7].

Table 1
Recovery (%) and precision (R.S.D., %) data in assaying the influence of TCA concentration on CTC and 4-epi-CTC isolation from pig kidney samples ($n = 6$)

Concentration of TCA (%)	Analytes	
	CTC	4-epi-CTC
10	20 (11)	25 (10)
20	53 (9)	57 (11)
30	73 (7)	75 (9)
40	70 (11)	78 (12)
50	68 (10)	79 (10)

In this studies, two extraction procedures we compared: (a) the previously described procedure [20] with combination of 0.1 M oxalic acid and acetonitrile for extraction/deproteinization followed by dilution with oxalic buffer, (b) 0.1 M oxalic buffer (pH 3.5) and 30% TCA solution for extraction/deproteinization followed by additional homogenisation with 0.1 M oxalic buffer. The preliminary studies indicated that using the extraction procedure with 0.1 M oxalic acid and acetonitrile is not suitable for simultaneous assaying CTC and 4-epi-CTC because of variable recoveries from kidney samples. More optimistic results were obtain, when biological matrix was treated with 0.1 M oxalic buffer and 30% TCA solution. The best assay conditions were found when 0.1 M oxalic buffer was added slowly with continual stirring. Further, 30% TCA solution was added drop-by-drop and stirred all the time. The procedure worked well, when additional aliquot of 0.1 M oxalic buffer was added and the sample was homogenised with added solutions. The optimal deproteinization effect was obtain on using 30% TCA solution (Table 1).

The experiences with the environment of spiking solutions and the time of exposition indicated that spiking procedure have important influence on the recovery of the analytes from biological matrix (Table 2). The oxalic buffer protects CTC and its epimer from chelation with metal ion and binding to proteins from biological samples.

Table 2
Recovery (%) and precision (R.S.D., %) data in assaying the influence of spiking procedure on CTC and 4-epi-CTC isolation from pig kidney samples ($n = 6$)

Spike contact, time (min)	Spike solvent	Analytes	
		CTC	4-epi-CTC
0	Water	63 (8)	68 (11)
	Oxalic acid	70 (9)	74 (9)
	Methanol	72 (7)	71 (10)
15	Water	60 (9)	64 (12)
	Oxalic acid	73 (8)	74 (9)
	Methanol	70 (11)	69 (13)
60	Water	60 (13)	66 (14)
	Oxalic acid	72 (11)	73 (9)
	Methanol	62 (14)	61 (13)

Table 3
Recovery (%) and precision (R.S.D., %) data in assaying the influence of extraction cartridges on CTC and 4-epi-CTC isolation from pig kidney extracts ($n = 6$)

SPE cartridges	Analytes	
	CTC	4-epi-CTC
C_{18}	36 (11)	42 (13)
C_8	57 (14)	62 (13)
Strata X	96 (6)	98 (7)
Oasis HLB	94 (9)	92 (10)
SDB1	88 (11)	90 (12)

3.3. SPE optimisation

Five different SPE disposable columns have been tested for clean-up and preconcentration. In order to select a suitable cartridge, the commercially available modified polymers (Strata X, SDB1, and Oasis HLB) and silica (C_8 and C_{18}) sorbents have been compared.

To eliminate the extraction step as variable, the row extracts were spiked with CTC and 4-epi-CTC working standard and loaded to SPE cartridges. The volume and composition of eluting solutions were evaluated for the optimisation of the SPE procedure.

Generally, CTC and 4-epi-CTC were strongly adsorbed on all the cartridges after loading in solution with low pH environment. Two elution volumes were compared, 3 and 5 ml, and using 3 ml of methanol was sufficient for quantitative elution of the analytes. The use of higher volume or acid modifier did not affect significantly the final recovery rates, but endogenous compounds were co-eluted at higher amount and the obtained extracts were more "dirty".

As it was shown in Table 3, the recoveries from silica-based supports were markedly lower than those from polymer one. In this study, the removal of endogenous compounds using the Strata X cartridges was more effective than by other one. Hence, the use of this kind of the cartridges was found suitable for clean-up purposes.

3.4. Validation method

We were developing the simple and rapid sample preparation for the simultaneous determination of CTC and 4-epi-CTC in pig kidneys. The preliminary, comparative studies indicated that using composition of 0.1 M oxalic buffer and 30% TCA solution followed by SPE clean up with Strata X disposable SPE cartridges gives high and comparable recovery of the analysed compounds. The whole procedure was validated according to the requirements of the European Union regulation 2002/657/EC.

Six-point matrix-matched calibration curves were obtained by spiking blank kidney extracts with CTC and 4-epi-CTC in concentrations from 0.1 to 3.0 μg/ml. The linear regression coefficients (r^2) were 0.9982 and 0.9978 for CTC and 4-epi-CTC, respectively.

The specificity was evaluated by the analysis of 20 blank samples taken from the pig kidney. The chromatograms obtained from the analysis of the blank kidney extract and the spiked kidney extract are shown in Fig. 2b and c, respectively. No interfering peaks from endogenous compounds were found in the retention time of the target compounds.

Repeatability (precision) and recovery (trueness) of CTC and 4-epi-CTC were measured in blank pig kidney that were spiked at MRL level (600 μg/kg), at half the MRL level (300 μg/kg) and one and half the MRL level (900 μg/kg). The spiked samples were analysed and the recoveries were calculated by comparing the measured concentration to the spiked concentrations. The results of the studies are presented in Table 4. Recoveries were 70–75% with repeatability less than 20%, which fulfils the legislation requirements.

According to concept of the regulation 2002/657/EC, the CCα (decision limit) and CCβ (detection capability) have been estimated. CCα was calculated after analysis of 20 blank pig kidney samples spiked at level of 600 μg/kg (1 × MRL). The determined values of the CCα for CTC and 4-epi-CTC were 674.8 and 683.6 μg/kg, respectively. CCβ was calculated after analysis of 20 blank pig kidney samples spiked

Table 4
Repeatability and recovery of CTC and 4-epi-CTC determined in spiked pig kidney ($n = 6$)

	Added					
	300 μg/kg		600 μg/kg		900 μg/kg	
	CTC	4-epi-CTC	CTC	4-epi-CTC	CTC	4-epi-CTC
Day 1						
Mean (μg/kg)	226	229	436	440	675	669
R.S.D. (%)	8.9	9.7	12.4	13.6	11.6	10.3
Recovery (%)	75	75	73	74	75	74
Day 2						
Mean (μg/kg)	207	216	416	451	655	642
R.S.D. (%)	9.8	10.5	11.4	12.8	9.7	9.5
Recovery (%)	70	72	70	75	72	71
Overall						
Mean (μg/kg)	216	223	426	448	665	656
R.S.D. (%)	9.4	10.1	11.9	13.2	10.6	9.9
Recovery (%)	72	74	71	74	74	73

at corresponding CCα level for both compounds. The determined values of the CCβ were 684.6 and 696.3 μg/kg, respectively.

The stability of the analytes was determined in solvent (stock solutions), in matrix (spiked samples at 600 μg/kg), and in eluate stored prior the LC analysis. The individual stock solutions prepared in methanol and stored at −20 °C were stable for 6 months. The stability of the combined stock solutions stored at 4 °C was 1 month, and working aqueous solution was stable for 2 weeks. The stability of the spiked sample stored at −20 °C was estimated for at least 4 weeks. The stability of eluate stored prior to the analysis was 2 days at ambient temperature.

The ruggedness (robustness) and applicability of the whole procedure developed for the simultaneous determination of CTC and its 4-epi-CTC in pig kidney has been evaluated. As minor changes of experimental conditions: changes like solvent, reagent bath temperature, and major changes like pH value, volume of reagents, spiking, and operator were evaluated. As it was found the critical points of sample preparation were pH value of oxalic buffer and volume and concentration of TCA solution.

4. Conclusions

The improved, rapid and simple extraction and clean-up procedure for the detection and determination of CTC and 4-epi-CTC in pig kidneys has been developed and can be used for residue control purposes. The obtained validation results indicate the accordance of the method performance with to the requirements of the European Union regulation 2002/657/EC.

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