DRUGS, COSMETICS, FORENSIC SCIENCES

Comparison of Micellar Electrokinetic Chromatography, Liquid Chromatography, and Microbiologic Assay for Analysis of Cephalexin in Oral Suspensions

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Two well-accepted methodologies, based on a microbiologic assay (MA) and liquid chromatography (LC), and a novel methodology using micellar electrokinetic chromatography (MEKC), were compared for the determination of cephalexin in commercially available and simulated samples of oral suspensions. The MA, described in the *Brazilian Pharmacopeia*, was performed with a strain of *Staphylococcus aureus* ATCC 6538 as the test organism, following the cylinder-plate method. The LC analysis followed the *European Pharmacopoeia*, 3rd Ed., and was used with minor modifications. The MEKC analysis was based on a previous work of the group. Estimates of the repeatability relative standard deviations of the MA, LC, and MEKC methods in the analysis of a commercial sample were 0.34, 0.42, and 0.37%, respectively. The recovery obtained with LC was 99.90 ± 1.11%; for MEKC, it was 100.09 ± 0.56%. Although the 3 methodologies were statistically equivalent for the determination of cephalexin in oral suspensions, MA gave suitable repeatability despite being nonspecific and time-consuming. MEKC provided faster analysis and higher column efficiency, whereas LC presented superior sensitivity. The results indicated that MEKC can be used as an alternative method to MA and LC in routine quality control laboratories.

Cephalosporins are a group of β-lactam antibiotics similar in structure and action to the penicillins. The compounds are used extensively in the treatment of Gram-positive and -negative infections. Cephalexin is a cephalosporin antibiotic, which presents an excellent oral absorption. It is widely used in pharmaceutical preparations such as tablets, capsules, and oral suspensions. The structural formula of cephalexin is given in Figure 1.

Many analytical methods have been described for cephalexin quantitative determination, including ultraviolet (1, 2) and visible spectrophotometry (3–7), spectrofluorimetry (8), iodometric titration (9, 10), microbiologic assay (10–13), liquid chromatography (LC; 12–24), and, more recently, capillary electrophoresis (CE; 25–29). However, only iodometric titration, microbiologic assay (MA), and LC are accepted by official codes for the analysis of cephalexin oral preparations (9, 11, 16, 21).

MA is a reference technique used for quantitative determination of antibiotics in pharmaceutical preparations because of its high sensitivity and suitable precision. Although MA is less accurate than instrumental methods, it is a unique method that can evaluate the intrinsic activity of cephalexin, considering that the degradation products do not have microbiological activity.

Most cephalosporins are often chromatographed without prior derivatization procedures. Good chromatographic separations can be obtained on C_8 or C_{18} materials, using simple mobile phases by adjusting, when necessary, the organic modifier concentration, the pH and, eventually, the flow rate. Mobile phases containing only one organic modifier and a phosphate buffer system are often preferred, giving satisfactory results in many cases (23). The *United States Pharmacopeia* indicates ion pair methodologies in reversed-phase chromatography to evaluate pharmaceutical preparations containing cephalexin.

In the last few years, CE has become a mature separation technique, increasingly used in routine analysis, with a number of successful applications in pharmaceutical sciences (30). CE instruments work reliably, and many new approaches and reagents have made method development easier. Good quanti-
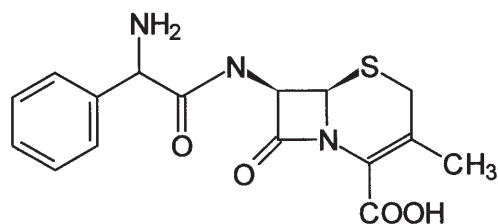
Figure 1. Chemical structure of cephalexin.

tative results have been obtained and validation guidelines outlined.

CE is actually a family of techniques, with distinct applicability toward the separation of compounds of different chemical character, size, and structural features. Among the members of the family, micellar electrokinetic chromatography (MEKC) is largely used in the analysis of neutral pharmaceuticals (31). In MEKC, a surfactant is added to the running buffer in a concentration higher than its critical micelle concentration, generating a pseudo stationary phase, in which solute partition takes place.

MEKC has been widely used in the analysis of cephalosporin compounds (27, 28, 32). Its use was first described for the separation of 9 cephalosporins in a mixture containing sodium dodecyl sulfate (SDS) as ionic surfactant (28). The successful analysis of cephalexin is also reported in the literature (29). The method provided suitable resolution and selectivity between the drug and its degradation products.

For certain complex mixtures, however, a suitable surfactant might not be found, typically because of the lack of selectivity and/or a narrow elution window (33). In these situations, the use of mixed micelles can lead to enhanced separations. Nonionic long chain alkyl surfactants such as Brij and Tween in conjunction with SDS have been successfully tested for MEKC separations. The mixed micellar medium containing a mixture of ionic and nonionic surfactant (SDS/Brij 35) was used to quantify cephradine and cephalexin (26). The addition of the nonionic surfactant improved migration time reproducibility and resolution of the cephradine and cephalexin peaks. The same system was also used for quantitative analysis and preliminary study of thermal stability of cephalexin in pharmaceutical oral suspensions (34). Complete separation of the drug and degradation products was obtained after thermal stress of oral suspensions (34).

The fact that LC and CE operate on different separation principles makes these 2 techniques potentially complementary to each other. The analysis of complex structures exhibiting a broad spectrum of physicochemical properties, as is often the case in pharmaceutical preparations, may be feasible using one or both techniques (35). Despite their apparent differences, combinations of CE and LC are frequently used in cross-validation studies during method validation (36).

This study presents a statistical comparison of performance for a novel MEKC method and 2 analytical methods (LC and MA) described in official codes for determination of cephalexin in commercially available and simulated oral suspensions.

Experimental

Standards

(**a**) *Cephalexin.*—Eli Lilly (São Paulo, Brazil).

(**b**) *Acetaminophen internal standard (IS).*— Janssen-Cilag (São Paulo, Brazil).

Solutions

(**a**) *Stock solutions.*—Aqueous standard stock solutions of cephalexin (200 μg/mL) and acetaminophen (200 μg/mL) were prepared.

(**b**) *Working solutions.*—Appropriate concentrations were prepared daily by diluting stock solutions in water for both CE and LC analysis.
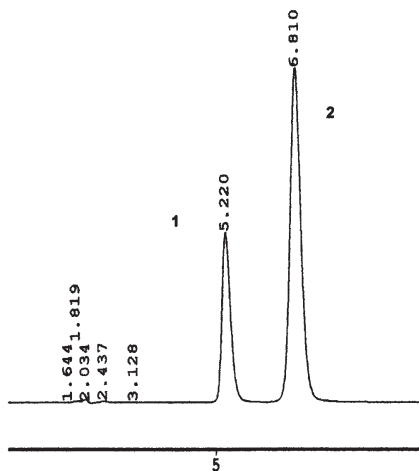

Figure 2. LC analysis of cephalexin: (1) acetaminophen (internal standard); (2) cephalexin.
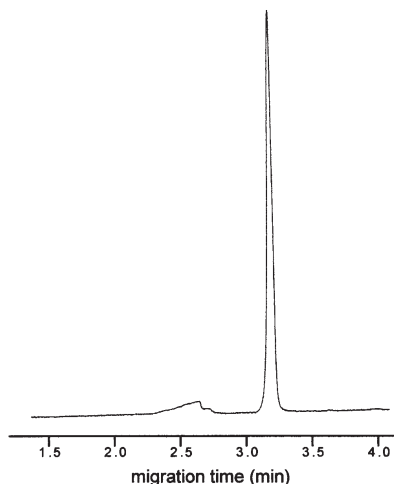

Figure 3. MEKC analysis of cephalexin (80 μg/mL).

Table 1.   Suitability test parameters of LC and MEKC methods for analysis of cephalexin

Parameter	Method	
	MEKC	LC
Efficiency, N	25421	1147
Retention factor, k	2.21	2.31
Retention time, min	3.41	6.80
Peak asymmetry	0.99	1.05

Samples

(**a**) *Sample 1.*—Commercially available oral suspension containing 25 mg/mL cephalexin was supplied by a local pharmaceutical industry.

(**b**) *Sample 2.*—Simulated sample of oral suspension containing 25 mg/mL cephalexin and excipient in sufficient quantity (38 g) was also supplied by a local pharmaceutical industry. Both samples were prepared with the following constituents: cephalexin (1.5 g), strawberry flavor (95.0 mg), sodium erithrosine (4.0 mg), and sucrose (36.4 g).

Microbiological Assay

(**a**) *Microorganism.*—*Staphylococcus aureus* (ATTC 6538P).

(**b**) *Inoculum.*—0.1% (v/v).

(**c**) *Diluent.*—Phosphate buffer solution 0.1M (pH 6.0).

(**d**) *Assay conditions.*—The protocol of analysis followed the *Brazilian Pharmacopeia* (4th Ed.), using the cylinder-plate method, which is based upon diffusion of antibiotics from a vertical cylinder through an agar layer (7). Stock solutions of standards and test samples (1 and 2) were diluted to 250 µg/mL with water and then diluted to 5, 10, and 20 µg/mL with phosphate buffer solution (pH 6.0) on the day of analysis. Three alternated cylinders were filled with 200 µL of the reference concentration solutions and the other 3 cylinders with sample solutions. Nine plates of 10 cm id were used for each sample. After a 16 h incubation, the inhibition zone diameter (mm) was measured by a digital caliper (Mycal CD-6CS, Tokyo, Japan). Percentage of cephalexin on each sample was calculated according to the Hewitt equation (37).

Liquid Chromatography

(**a**) *LC system.*—An LC Shimadzu (LC 10 AD) equipped with a variable UV detector SPD-10A automatic injector Model Sil 10A and an integrator were used.

(**b**) *Column.*—An   analytical   column   LiChrospher® (Merck, Darmstadt, Germany) 100 RP 18 (5 µm) was used.

(**c**) *LC conditions.*—All experiments were conducted under isocratic conditions at room temperature (24 ± 1°C). The mobile phase flow rate was 0.8 mL/min and the sample injection volume was 20 µL. The UV detector was set at 260 nm.

(**d**) *Eluent.*—Acetonitrile–0.013M phosphate buffer (8 + 92, v/v).

(**e**) *Ultrasonic apparatus.*—Thorton Model T14, with 40 kHz frequency (São Paulo, Brazil).

(**f**) *Filtering system.*—Filter Durapore (Millipore®, Bedford, MA), GVWP 04700, 0.22 µm for eluent filtration; filter Durapore (Millipore) GVWP 01300, 0.45 µm for sample filtration.

(**g**) *pH meter.*—Equipment capable of measuring pH to 0.01 units (Model TE-901 Digimed, São Paulo, Brazil).

(**h**) *Sample preparation.*—Nine flasks of each sample (1 and 2) were used. Amounts corresponding to 250 mg cephalexin of each sample were weighed, transferred into 250 mL volumetric flasks, and diluted to volume with water. Aliquots (4.0 mL) of these solutions were transferred into 50 mL volumetric flasks and diluted to volume with water. Acetaminophen was used as IS. A standard solution was pre-

Table 2.   Statistical parameters of the calibration curve for MA, LC, and MEKC methods in analysis of cephalexin

Statistical data	Cephalexin		
	MA	LC	MEKC
Concentration range, µg/mL	5–20	40–120	40–120
Intercept[a]	0.762	0.016	27.57
Slope[a]	0.083	0.027	3.560
Correlation coefficient, R^2	0.9980	0.9999	0.9990
Relative standard deviation, %			
Retention/migration time	—	0.24	1.18
Peak area	—	—	0.43–1.71
Peak area ratio	—	0.09–0.19	—
Inhibition zone diameter	1.72–3.07	—	—
Limit of detection, µg/mL	—	1.68	2.39
Limit of quantitation, µg/mL	—	5.11	7.95

[a] Data obtained from standard curve.

Table 3. Quantitative analysis of cephalexin in commercially available sample (1) and simulated sample (2) of oral suspension, using MA, LC, and MEKC methods

	Cephalexin oral suspension sample	
Parameter	1	2
MA[a]		
Amount declared, mg/mL	25.00	25.00
Amount found, mg/mL	24.89	25.20
Standard deviation (s)	0.82	0.90
RSD,[b] %	0.34	0.37
LC[c]		
Amount declared, mg/mL	25.00	25.00
Amount found, mg/mL	24.91	25.28
Standard deviation (s)	0.36	0.58
RSD, %	0.37	0.57
MEKC[c]		
Amount declared, mg/mL	25.00	25.00
Amount found, mg/mL	24.99	24.99
Standard deviation (s)	0.42	0.64
RSD, %	0.41	0.64

[a] Average of 6 samples.
[b] RSD = Relative standard deviation
[c] Average of 9 samples.

pared at the same concentration of the sample, following the procedure described above.

Capillary Electrophoresis

(**a**) *CE system.*—Perkin-Elmer Model 270A-HT (Foster City, CA), equipped with variable UV-Vis detector. The instrument was operated under positive polarity (injection end of capillary).

(**b**) *Capillary column.*—An uncoated fused silica capillary (Polymicro Technologies, Phoenix, AZ), 75 μm id and total length 50 cm (28 cm to detector), was used. The capillary column was flushed for 30 min with filtered 1M NaOH solution, 15 min with deionized water, and 30 min with the buffer electrolyte.

(**c**) *CE conditions.*—A stock solution of 100mM sodium tetraborate was degassed in an ultrasonic bath and filtered through a 0.22 μm membrane filter (Millipore) before use. The electrolyte solution was constituted by 20mM SDS, 0.1% lauryl polyoxyethylene ether (Brij 35), and 20mM sodium tetraborate buffer, pH 9.23. The electrolyte buffer was prepared at the beginning of the day. Hydrodynamic injection of samples was performed with Hg at 5 in./5 s. A constant voltage of 15 kV was used for all experiments.

Between runs, the capillary was rinsed with deionized water for 1 min, followed by electrolyte buffer for 3 min. The UV detector was set at 210 nm and the temperature was set at 30°C. Data acquisition and treatment software (Turbochrom®,

PE-Nelson, Cupertino, CA) was used for peak integration and data analysis.

(**d**) *Sample preparation.*—Nine flasks of each sample (1 and 2) were used. Amounts corresponding to 250 mg cephalexin were weighed, transferred into 250 mL volumetric flasks, and diluted to volume with water. Aliquots of 4.0 mL of these solutions were transferred into 50 mL volumetric flasks and diluted to volume with water. A standard solution was prepared at the same concentration of the sample, following the procedure described above.

Linearity

(**a**) *LC.*—Appropriate aliquots of the standard stock solutions of cephalexin and acetaminophen were transferred separately into 10 mL volumetric flasks and diluted to volume with water. Concentration ranges from 40 to 120 μg/mL cephalexin, with 20 μg/mL acetaminophen, were obtained. Each solution was injected in triplicate. Peak area ratios (cephalexin/acetaminophen) were plotted vs the respective concentrations of cephalexin.

(**b**) *MEKC.*—Appropriate aliquots of standard stock solutions of cephalexin were transferred separately into 10 mL volumetric flasks and diluted to volume with water. Concentration ranges from 40 to 120 μg/mL cephalexin were obtained. Each solution was injected in triplicate. Peak area counts were plotted against the respective concentrations of cephalexin.

Precision

The precision of MA, LC, and MEKC methods were evaluated from relative standard deviation (RSD) of each point of the calibration curve and the analysis of cephalexin in the samples. Within-day variability of sample 1 was obtained by analysis of 6 replicate samples using the MA and 9 replicate samples using the MEKC and LC methods.

Table 4. Percentage values of cephalexin in commercially available sample, using MA, LC, and MEKC methods

MA[a]	LC[b]	MEKC[b]
100.06	99.23	100.37
99.80	100.11	99.63
98.80	100.31	100.13
100.81	99.34	100.58
99.44	99.69	99.65
98.59	99.54	99.37
—	99.45	100.35
—	99.33	99.69
—	99.72	100.16

[a] Average of 6 samples.
[b] Average of 9 samples.

Table 5. Analysis of variance of quantitative determination of cephalexin in commercially available oral suspension sample based on results in Table 4

Source of variation	Degrees of freedom	Sum of squares	Mean of squares	F^a
Between methods	2	0.81	0.40	1.45
Error	21	5.86	0.28	
Total	23	6.68		

[a] Tabulated Fischer F value with 95% confidence level and 23 degrees of freedom, $F = 3.42$.

Accuracy

To determine accuracy of the MEKC method, recovery experiments were performed according to AOAC guidelines (38). Commerical samples containing a fixed amount of cephalexin (400 µg) were spiked with aliquots of 1, 2, and 3 mL of a standard cephalexin solution (200 µg/mL) in separate 10 mL flasks. The final cephalexin concentrations obtained were 60, 80, and 100 µg/mL, respectively. For analysis of cephalexin by LC, the same procedure as described above was followed, and acetaminophen (20 µg/mL) was used as IS. The samples and the standard solution were filtered using a 0.22 µm filter before injection.

Specificity

The specificity of the LC and MEKC methods was evaluated as described on official codes and some reports (39–41). The interference of inactive ingredients was evaluated from placebo sample of oral suspension prepared with the same constituents as those of sample 1.

Limits of Detection and Quantitation

Limits of detection (LOD) and quantitation (LOQ) were calculated from the standard deviation (SD) of response and slope curve (S) in accordance with the equations LOD = 3.3 (SD/S) and LOQ = 10 (SD/S; 39, 41, 42).

Results and Discussion

Separation Characteristics

The development of a novel analytical method demands comparison of its performance with other methodologies already established in official codes.

The LC method for cephalexin listed in the *European Pharmacopoeia* (16) indicates the use of a mobile phase containing acetonitrile, methanol, and phosphate buffer. Preliminary evaluations showed that average retention time for cephalexin with this mobile phase was 12 min. However, retention times of acetaminophen and cephalexin can be considerably reduced when a mobile phase containing only phosphate buffer and acetonitrile is used, as depicted in Figure 2. Therefore, this modified mobile phase composition was used throughout the study.

Figure 3 shows an electropherogram of cephalexin obtained from the MEKC methodology developed with a mixed micellar medium. The performance parameters of the chromatographic and electrophoretic methods are given in Table 1. As shown in Table 1, column efficiency of the MEKC method is higher and the analysis is faster, with better peak symmetry.

Cross-Validation

The linearity of a specific method is defined as the ability of the method to elicit test results that are directly proportional to the analyte concentration within a given range. Statistical data derived from the regression line of the 3 analytical methods compared in this work are evaluated in Table 2. The 3 methodologies present fairly good linearity in the range of concentration studied, with acceptable correlation coefficients for analytical purposes. LC presented better repeatability of retention time and better precision for peak area because an IS was used. MA repeatability of inhibition zone diameter was reasonably good with RSD <3%. An enhanced response was obtained with MEKC for the same range of concentration studied in LC, although with a much higher value of intercept. LC presented lower LODs and LOQs.

Table 3 shows the results of the analysis of oral suspensions of cephalexin by the 3 methodologies. Usually, biological assays present higher RSD values in the analysis of drugs.

Table 6. Recovery test based on addition of standard solution of cephalexin to commercially available sample

Method	Standard added, µg/mL	Standard found, µg/mL	Recovery, %[a]	Average of recovery, % ± s
LC	20	19.77	98.88	
	40	40.78	101.96	100.55 ± 1.55
	60	60.48	100.80	
MEKC	20	20.14	100.71	
	40	39.98	99.94	100.09 ± 0.56
	60	59.77	99.62	

[a] Each value is average of 3 determinations.

Table 7. Comparison of accuracy and precision between LC and MEKC methods in analysis of cephalexin oral suspensions

Samples[a]	Accuracy, calculated t value[b]	Precision, calculated F value[b]
1	1.92	0.82
2	0.79	1.22

[a] Sample 1: commercially available sample of cephalexin oral suspension; sample 2: simulated sample of cephalexin oral suspension.

[b] Tabulated Student t value with 95% confidence level and 16 degrees of freedom, $t = 2.11$; tabulated Snedecor F value with 95% confidence level, $F_{8/8} = 3.44$.

However, the precision of the MA was very similar to that of the instrumental techniques.

Table 4 shows the results of the quantitative analysis of cephalexin in a commercially available oral suspension sample by the 3 methodologies. The results indicated a fairly good agreement. Statistical tests were performed to ensure that the results were equivalent. An analysis of variance (ANOVA) was used to detect significant differences among the results of cephalexin analysis as determined by the 3 analytical methodologies (Table 5). The ANOVA of the values did not indicate significant differences among the 3 methods considering $p < 0.05$ (Table 5). This fact points out that MEKC can be used

as an alternative method to MA and LC to quantify cephalexin samples with a high degree of reliability.

Accuracy of analytical method is defined as the closeness of agreement between the value found by the method and the value that is accepted either as a conventional true value or as a reference value (39). The method accuracy was evaluated from the recovery of different amounts of standard cephalexin solution added to the commercially available sample (Table 6). The results indicated that LC and MEKC gave similar recoveries.

The accuracy and precision of the LC and MEKC methods were compared by the t and F tests, respectively (40, 43), taking into consideration the analysis of the commercial and simulated samples. The calculated t and F values are presented in Table 7 for $p < 0.05$. As indicated in Table 7, there is no statistically meaningful difference between the methods in terms of accuracy and precision. This observation is in agreement with those of other authors who evaluated capsules of cephradine (26).

The specificity of the chromatographic and electrophoretic methods is demonstrated in Figures 4 and 5, which present the analysis of the 2 samples of cephalexin and corresponding placebos. As demonstrated, the excipients of the suspension do not interfere with the cephalexin analysis; the placebo sample shows no peaks during analysis time of cephalexin.

Conclusions

The results demonstrate that the proposed MEKC methodology can be used to quantify and analyze cephalexin in oral
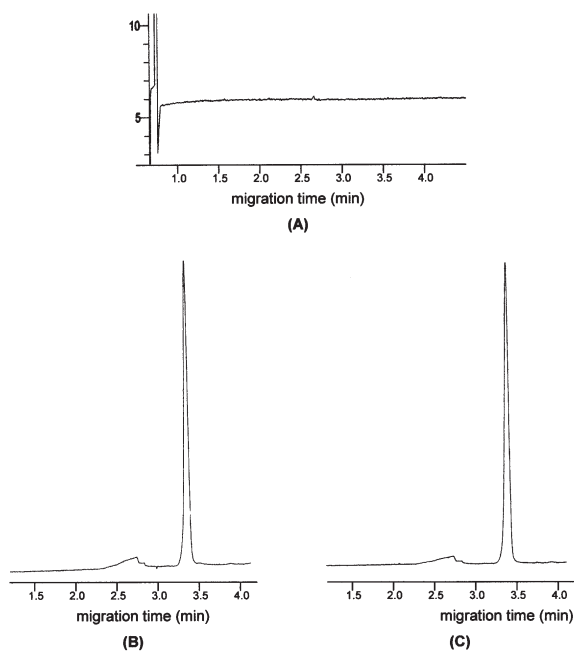
Figure 4. Specificity test of MEKC method in analysis of samples of cephalexin oral suspensions: (A) placebo from simulated sample; (B) commercial sample (oral suspension), 80 μg/mL; (C) simulated sample (oral suspension), 80 μg/mL.
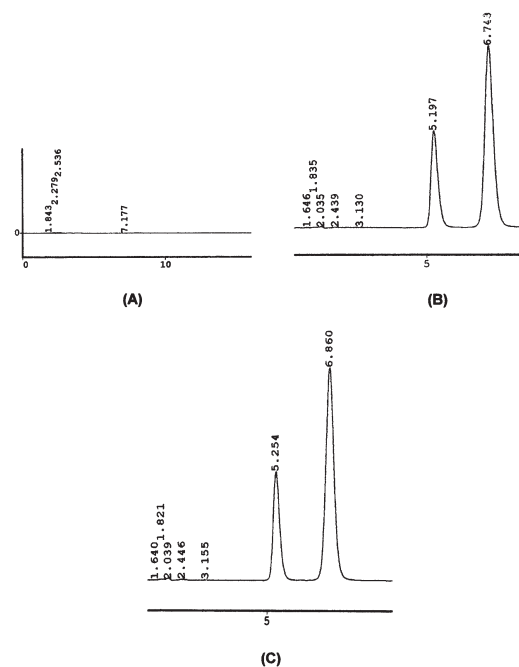
Figure 5. Specificity test of LC method in analysis of samples of cephalexin oral suspensions: (A) placebo from simulated sample; (B) commercial sample (oral suspension), 80 μg/mL; (C) simulated sample (oral suspension), 80 μg/mL.

suspensions as an alternative to LC and MA official methods. Moreover, important characteristics such as simplicity, low cost, and the capacity to analyze groups of different pharmaceuticals make CE suitable for quality control laboratories in the analysis of complex pharmaceutical samples.

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