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A New, Simple Colorimetric Method For Assaying Diosmin, And Flavonoids In Daflon Tablets And Orange Peel Extracts

S. A. Mir*, A. A. Ahangar, A. S. Bhat

Division of Veterinary Pharmacology & Toxicology, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, F.V.Sc. & A.H., Shuhama, Srinagar-190001, J & K, India

***Corres. author: mirsamir.19@rediffmail.com**
Telephone/Telex No: 91-0194-2262216

Abstract: A new colorimetric method for determination of diosmin is proposed. The method is based on formation of a chromophore by the flavonoid in presence of 75% (v/v) HCl showing maximum absorbance at 405 nm. The technique with linearity over 10 through 80µg flavonoid (r ± s.e. = 0.9994 ± 0.0008, b±s.e. = 0.0089 ± 0.0001, COV ± s.e. = 2.8 ± 0.5, n= 6 each) has been compared with standard aluminium chloride method and a modified version that utilizes aluminium chloride for primary complexation of the flavonoid, and then its determination by HCl method. The three techniques have been compared for determination of diosmin-equivalent flavonoid in Daflon tablets, assaying recovery of added diosmin to daflon extract, and determination of flavonoid in orange peel extracts. At approximately equi-normal concentrations perchloric and HCl were found most effective than sulfuric and nitric acids whereas o-phosphoric was least effective and glacial acetic acid ineffective in inducing the chromophore. Phenolics like phenol, hydroquinone, resorcinol, catechol, guaiacol, pyrogallol, gallic acid and tannic acid fail to develop color in absence or presence of glucose. This indicated that phenolic constituents and carbohydrate moiety in the flavonoid are not participating in the formation of chromophore.
Key-words: Spectrophotometric assay, Flavonoid, Diosmin, Daflon, Orange peel.

INTRODUCTION

Flavonoids are valuable anti-oxidants widely distributed in plants. Of these, diosmin, hesperidin and rutin are therapeutically useful drugs. Flavonoid analysis in botanicals and pharmaceuticals has been accomplished by employing a variety of techniques including colorimetric[1-13], chromatopgraphic[14-21] and others[22-25]. Diosmin occurs naturally in some botanicals (citrus fruits, vetch and hyssop) and is an established phlebotropic agent with diverse pharmacologic and therapeutic potential including utility in chronic venous insufficiency, hemorrhoids, lymphedema, diabetes, cancer and other clinical disorders[26]. Diosmin and its close congener hesperidin, a major semi-synthetic source for diosmin, have been demonstrated in orange peels along with neohesperidin and naringin[27]. The two flavonoids, diosmin and hesperidin, are pharmaceutically available for therapeutic use in the form of Daflon tablets.

Generally colorimetric methods for flavonoids are considered reliable, accurate and time-saving compared to chromatographic methods that though superior require advanced expertise and instrumentation, and are time-consuming and expensive, and therefore, remain beyond the reach of most laboratories[8]. Aluminium chloride method is most often the colorimetric method of choice for general determination of flavonoids [1, 3-11].

A need to standardize a new colorimetric method for determination of flavonoids was mooted on the basis of a serendipitous observation whereby in presence of high concentration of hydrochloric acid, many flavonoids including diosmin, daflon (mixture of diosmin and hesperidin), rutin, quercetin, morin and even extracts prepared from flavonoid rich peels from citrus fruits and tobacco leaves reacted uniformly with an intense yellowish coloration in concentration dependent manner. This necessitated systematic evaluation and optimization of the method, and its comparison with standard aluminium chloride method. A modified version of the method was also mooted that employed initial complexation of aluminium and flavonoid followed by subsequent determination of flavonoid by hydrochloric acid method. The present study focused on comparison of the techniques for assaying diosmin followed by assay of flavonoids in daflon tablets and in orange peel extracts in terms of standard diosmin.

MATERIALS AND METHODS

The experiments were carried out at an ambient temperature of 26.9 $\pm 0.5^0$C. The chemicals and drugs used were of standard quality obtained from reputed sources in India. Spectrophtometric measurements were initially made with available ordinary spectrophotometer (UV-Visible, Model SL-150, Elico (India) Ltd). Later studies as provided in this manuscript have been carried out with an improved model (UV-Visible, Model UVmini-1240 (Shimadzu Corporation, Japan)).

Reagents

Acids: Concentrated HCl and perchloric acid were estimated to have mean normality strength equal to 11.2N and 12.0 N by neutralization titrimetry. Other acids employed for the study included concentrated nitric acid, sulfuric acid, o-phosphoric acid and glacial acetic acid. The acids were approximated to strength of 75% HCl in water by appropriate dilution with water.

Aluminum chloride solution: 0.1 M solution was prepared by dissolving 2.5 g aluminium chloride hexahydrate in water to make 103.5 mL volume.

Potassium acetate 1 M: Prepared by dissolving 10 g potassium acetate in water to make 101.8 mL solution.

Sodium hydroxide solution: A 10 %(w/v) solution of sodium hydroxide pellets was made in water to provide a stock of 2.5 M NaOH solution. Appropriate working solutions were made by dilution with water.

Phenolics: Catechol, resorcinol, phenol, hydroquinone, gallic acid, pyrogallic acid and tannic acid were each made 0.2 % (w/v) in water. Guaiacol was prepared as 1% (w/v) in 10% ethanol.

Acetic acid solution: A 10% (v/v) solution of glacial acetic acid in water.

Ammonia solution: 5 % (v/v) ammonia in water (stock 25% diluted 5-folds).

Ammonium chloride solution: 1 M ammonium chloride solution was made by dissolving 13.5 g ammonium chloride in water to make 250 mL volume.

Glucose: 1 % (w/v) in water.

Standard diosmin

Synthetic diosmin purified from Venex-500 tablets (Elder Pharmaceuticals Ltd., Mumbai) served as a Laboratory standard. The tablets with gross weight 1032 ± 5 mg table^{-1} (n=9) were finely pulverized with pestle and mortar. Each tablet was labeled to contain 500 mg synthetic diosmin (B.P.). Each 207 mg gross tablet powder, equivalent to 100 mg synthetic diosmin, was added 20 ml distilled water, extracted at room temperature for one hour, and filtered over Whatman Filter No. 1. The residue was washed with about 50 mL water to remove water soluble impurities and coloring excipients, and then reconstituted with 10 ml water, and added 4 ml 2.5 N NaOH to solubilize diosmin, and volume was made 50 ml with water. The sample was filtered, and the filter paper washed with water to get 100 ml volume to get 0.1 % (w/v) labeled synthetic diosmin in 0.1 M NaOH. Further dilutions made in 0.1 N NaOH as per need. The stock solution was stored well stoppered in refrigerator. Diosmin was quite stable in alkaline medium, and when kept stoppered when not in use. Samples of diosmin on exposure to air or on rendering acidic developed opalescence.

Daflon flavonoid mixture

Daflon-500 tablets (Serdia Pharmaceuticals Ltd., Mumbai) were employed for assaying their flavonoid content by the test techniques. Each film coated tablet contained 500 mg bioflavonoids as per label (450 mg diosmin and 50 mg hesperidin). The tablets (gross weight 683 ± 3 mg, n=9) were finely pulverized to a homogeneous mass. One hundred mg flavonoid equivalent powder was processed just like Venex-500 to get 0.1 % (w/v) labeled flavonoid in 0.1 M NaOH. Dilutions made in appropriate solvent as per need.

Orange peel extracts

Purified ethanolic orange peel extract

The peels, collected from oranges obtained from local market, were gently washed with distilled water, dried between folds of filter paper, air-dried in shade over a week, and finely pulverized in pestle and mortar. Powdered peels were extracted over about 40 hours at room temperature to prepare 0.4 % (w/v) powder in 0.1 N NaOH. The mixture was filtered over Whatman Paper No. 1 and each 50 mL filtrate was neutralized with 10% HCl to pH around 7.0, the volume was reduced on sand-bath to about 5 mL, and finally made to 10 mL volume with water, added 40 mL ethanol, extracted for 10-15 minutes, filtered through Whatman No.1. An aliquot of filtrate, 40 mL of 0.4 % peel powder in 80% ethanol, was again reduced to 2 mL on sand-bath, added 4 mL 1M NaOH and volume made 40 mL with water to get aqueous alkaline extract, 0.4 % in 0.1N NaOH. The aqueous extract was added 20 mL each of organic solvents: chloroform, benzene and petroleum ether, vigorously shaken, allowed to stand half an hour for complete extraction, filtered and aqueous phase emerging first collected, kept in hot water at 80^0C to remove organic impurities, and the volume adjusted to 0.2% (w/v) orange peel powder in 0.1 N NaOH. This constituted purified ethanolic orange peel extract (PEOPE).

Purified methanolic orange peel extract

Orange peel powder 5 g was extracted with 100 mL methanol over about 40 hours, filtered over Whatman No.1, marc extracted twice with 50 mL each portions of methanol, and methanolic extracts pooled to get 2.5 % (w/v) orange peel powder in methanol. Each 160 mL portion was added 40 mL water, volume reduced to 40 mL on sand-bath to eliminate methanol. The extract was added 20 mL each of organic solvents; benzene, chloroform and petroleum ether, vigorously shaken, allowed standing 10-15 minutes, filtered over Whatman No.1, and first emerging aqueous phase was collected, kept in hot water bath at 80^0C to remove organic solvent impurities, cooled to room temperature, and volume adjusted with alkali solution to provide 0.3%(w/v) powder in 0.1N NaOH. This constituted purified methanolic orange peel extract (PMOPE).

Response of diosmin to test acids

One milliliter flavonoid solution containing 50 µg diosmin in 0.1 N NaOH was added each 3 mL of concentrated HCl and other acids made to 3 mL with water including perchloric acid (3 mL), sulfuric acid (1 mL), nitric acid (2.7 mL), o-phosphoric acid (2.2 mL) and glacial acetic acid (2 mL) approximately equal to the strength of 75 % HCl in 4 mL reaction volume. The samples were mixed and allowed to stand at room temperature for about 40 minutes, and read spectrophotometrically at 405 nm.

In a second set of experiments, a comparative evaluation of perchloric and hydrochloric acids over 0.5 through 3.0 mL (approximate acidity range 1.4 through 9.0 N) with 50 µg diosmin in a 4 mL sample volume was monitored at 405 nm to find relative action of the two acids on color development.

Response of phenolics in absence and presence of glucose to concentrated HCl

Each one milliliter sample solution containing 1 mg of test phenolic including phenol, catechol, resorcinol, hydroquinone, guaiacol, gallic acid, pyrogallol or tannic acid in absence or presence of glucose up to 2 mg were added 3 mL concentrated HCl, and color monitored at 405 nm following about 40 minute standing at room temperature.

Analytical techniques for flavonoids

Standard aluminium chloride method

Each milliliter of test solution in 0.1 N NaOH was added 0.4 mL of 10% (v/v) acetic acid in water followed by addition of 1.0 mL of 0.1 M $AlCl_3$ solution. The samples were added 1.6 mL 1 M potassium acetate solution, and color monitored at 385 nm at 15 to 20 minute standing at room temperature. Control samples contained all

reagents substituting aluminium chloride solution by addition of 1.0 mL water, and the acid was added just before reading else it induced opalescence in absence of aluminium chloride.

Standard HCl method

Each milliliter of test flavonoid in 0.1 N NaOH was added 3 mL of HCl. The mixture was allowed standing for 30-40 min at room temperature, and then monitored spectrophotometrically at 405 nm against reagent blank containing 1 mL 0.1 N NaOH and 3 mL HCl.

Aluminium chloride – HCl method

Each milliliter of test sample was added 1 mL 10 % acetic acid and 1 mL 0.1M $AlCl_3$ solution. The mixture was allowed standing 5 minutes to allow complexation of the flavonoid with aluminum ions. The samples were mixed up and added 2 mL 5% (v/v) ammonia solution followed by 1 mL 1 M NH_4Cl. The samples were again mixed up and allowed standing 10 min to allow precipitation of aluminum-flavonoid complex. Each sample was added 4 mL water, thoroughly mixed up, centrifuged at 6000 rpm for 5 minutes to separate the precipitated flavonoid. The supernatant was discarded and the drained residue reconstituted in 1mL 0.1N NaOH was added 3 mL HCl. The color allowed to develop over about 40 minutes and monitored at 405 nm.

Comparative evaluation of optimized techniques

In the first phase of experiments, HCl method and its modified version were compared with standard aluminium chloride method using standard flavonoid diosmin over the concentrations range of 10 through 80µg in 1 mL aliquot. The assays were compared with respect to linearity, regression coefficients and coefficient of variation employing routine statistical procedures.

In the second phase of experiments, an aliquot of daflon extract (containing 30 µg total flavonoid mixture as per label) was assayed in terms of simultaneously run calibration curve for diosmin standard by using three optimized methods: HCl, $AlCl_3$ and $AlCl_3$ + HCl. Additionally, an aliquot of daflon solution containing 30µg of estimated flavonoid was added 30µg of diosmin in 1 mL volume, and the mixture was assayed for flavonoid content by the three methods to find recovery of added diosmin and per cent recovery. In the final phase of experiments, the techniques were applied to estimate content of flavonoids in purified extracts from orange peel powders in terms of diosmin standard.

RESULTS AND DISCUSSIONS

Effect of mineral acids and glacial acetic acid on diosmin

A serendipitous observation revealed a characteristic concentration-dependent yellow coloration of test flavonoid diosmin with concentrated hydrochloric acid. During preliminary observations and monitoring with an ordinary spectrophotometer that showed maximum absorbance at 430 nm, a similar characteristic observation was obtained with other test flavonoids including rutin, quercetin and morin. Glacial acetic acid and o-phosphoric acid failed to produce effect on 60 µg rutin while nitric and sulfuric acids were weaker compared to HCl and $HCLO_4$ when compared at normality approximated to the strength of 75 % HCl. Substitution of concentrated HCl by varying per cent of equi-normal sulfuric acid caused increase in absorbance with decrease in per cent substitution ($r= 0.999$, $b = 0.0027$, $n=4$ each, with respective substitution as 100, 67, 33 and 0 per cent by sulfuric acid). Availability of more sensitive spectrophotometer enabled more refined studies using diosmin as laboratory standard, and obtaining maximum absorbance at 405 nm. As apparent (Table 1) diosmin showed an appreciable response only with perchloric and hydrochloric acids compared to nitric, sulfuric and phosphoric acids whereas response was nil with glacial acetic acid. The relative order of potency between test acids at equivalent normality strength approximated to 75% HCl appeared as-

Perchloric acid ≥ Hydrochloric acid >> o-phosphoric acid ≥ sulfuric acid > nitric acid

These observations necessitated undertaking comparative evaluation of perchloric and hydrochloric over 0.5 through 3.0 mL (1.4 through 9.0 N acidity) with 50 µg diosmin in a 4 mL sample volume. The results revealed that the two acids induced perfectly linear increase in color intensity with increase in acidity , and perchloric acid was significantly more effective than hydrochloric acid at each tested concentration (Table 2) ($P<0.01$, $n= 5$ each). The ratio of regression coefficients obtained with the two acids, 1.45, is about 36 % more than the ratio of their relative mean acid strengths suggesting perchloric acid was definitely stronger in action than hydrochloric acid. This observation is consistent with the known relative strengths of the two acids whereby

perchloric and hydrochloric acids are considered to be, respectively as, *super and strong acids*. Hydrochloric acid was chosen in the present study for being a less hazardous acid to handle than perchloric acid.

Table 1: Effect of various acids on diosmin-dependent chromophore

Treatment	Absorbance	Mean acidity, N
Glacial acetic acid	Nil	9.0
Nitric acid	0.125 ± 0.008	9.0
Sulfuric acid	0.224 ± 0.005	8.8
o-phosphoric acid	0.230 ± 0.008	8.8
Hydrochloric acid	0.544 ± 0.009	8.4
Perchloric acid	0.649 ± 0.011	9.0

The values are mean ± s.e of four observations each.

Table 2: Comparative efficiency of hydrochloric and perchloric acids on diosmin dependent color intensity

Volume, mL	HCl[a]	$HCLO_3$[b]
0.5	0.024 ±0.003	0.051 ± 0.002
1.0	0.056 ± 0.001	0.115 ±0.004
2.0	0.297 ± 0.004	0.534 ±0.001
2.5	0.446 ± 0.002	0.616 ±0.006
3.0	0.533 ± 0.004	0.630 ±0.006
Statistical analysis[c]		
r± s.e.	0.9908 ± 0.0082	0.9847 ± 0.0150
b± s.e..	0.218 ± 0.013	0.3146 ± 0.0271

The values are mean± s.e of five observations each.

[a] HCl strength approximately equal to 11.20 ± 0.04 N

[b] $HCLO_4$ strength approximately equal to 12.03 ± 0.03 N

[c] The analysis with respect to $HClO_4$ over most linear range of data: 0.5 through 2.5 mL

Effect of HCl on phenolics in absence and presence of glucose

The color reaction of test flavonoids with HCl is apparently unrelated to the presence of phenolic residues and/or sugar component of the flavonoid. Glucose up to 2 mg in 1mL solution failed to react with the acid. In fact in a separate assay morin that lacks presence of sugar reacted like diosmin (and rutin) containing rutinose (a disaccharide of glucose and rhamnose). Similarly, 1 mg each of the test phenolics including resorcinol, guaiacol, catechol, phenol, gallic acid, pyragallol and tannic acid failed to react with 3 mL HCl in absence or presence of 1 mg of glucose. Therefore, these chemicals are unlikely to participate or interfere with the assay by HCl method. Resorcinol is a characteristic constituent of flavonoids such as morin, diosmin, hesperidin, rutin, quercetin and naringin with additional phenolic residues, respectively as, resorcinol, guaiacol, guaiacol, catechol, catechol and phenol.

Linearity response with test methods for diosmin assay

Three colorimetric methods exhibited perfect linearity over 10 through 80 µg of diosmin (Table 3). The mean absorbance values at all concentrations with HCl method was about 2-folds, 2.2 ± 0.2 (n=6), more than that obtained with $AlCl_3$ method as is evident from the ratio of their regression coefficients, about 2.3. The mean absorbance values with HCl method was almost comparable to the value obtained with $AlCl_3$ + HCl method as is evident from the ratio of their regression coefficients, 1.1. The coefficient of variation ranged from 2.8 to 5.4, and was better with HCl method, 2.8, than with $AlCl_3$ method, 4.5, or with $AlCl_3$ + HCl method, 3.9. The preliminary studies with rutin and morin at 430 nm had indicated similar results such as perfect linearity over 15 through 150 µg flavonoid (r= 0.99, n= 5 each), and the ratio of regression coefficients with HCl and $AlCl_3$ methods were correspondingly 1.24 and 1.36.

Acid method for determination of flavonoids offers a simple alternative method for colorimetric determination of flavonoids in test preparations. An improvised method was mooted on the consideration that aluminium ions show a high binding affinity with most flavonoids[28, 29], and this interaction enables use of aluminium chloride as a standard method for determination of flavonoids in test materials[8, 28, 30, 31]. An improvised method was mooted to use initial complexation of flavonoid and aluminium coupled to precipitation of aluminium-flavonoid complex in ammonia-ammonium chloride medium. Then acid method could be applied to the centrifuged residue. This improvised method is anticipated to improve more selective determination of flavonoid in presence of other possible interferences. This demanded evaluating effect of aluminium on flavonoid determination by HCl method. In presence of Al^{3+} ions, 10 through 250 µmoles, the mean absorbance values of diosmin and rutin remained unaffected (P>0.1, n= 3 each).

Table 3: Assay of diosmin by three methods

Diosmin , µg	Analytical methods		
	$AlCl_3$	HCl	$AlCl_3$ + HCl
10	0.049± 0.001	0.097 ± 0.002	0.090 ± 0.002
20	0.084 ±0.003	0.176 ±0.003	0.155 ± 0.002
30	0.139 ±0.002	0.272 ± 0.003	0.254 ± 0.006
40	0.160 ± 0.003	0.343 ± 0.002	0.305 ± 0.004
50	0.226 ± 0.002	0.449 ± 0.005	0.426 ± 0.007
80	0.313 ± 0.007	0.715 ± 0.006	0.579 ± 0.009
Statistical analysis			
r ± s.e.	0.9927 ± 0.0059	0.9994 ± 0.0008	0.9918 ± 0.0067
b ± s.e.	0.0038 ± 0.0002	0.0089 ± 0.0001	0.0072 ± 0.0004
COV ± s.e.	4.5 ± 0.8	2.8 ± 0.5	3.9 ± 0.4

The values are mean ± s.e. of six observations each

Assay of daflon extract and recovery of added diosmin

Daflon-500 tablets were chosen for assay as it contained dominantly diosmin, 90%, with remainder fraction hesperidin. Total flavonoid content was assayed in terms of standard diosmin. Assay of 30 µg of daflon-flavonoid mixture (as per label) with the three methods viz., HCl, $AlCl_3$ and AlCl3 + HCl revealed respectively, 30.88± 0.27, 30.42± 0.29 and 29.81 ± 0.46 µg of diosmin-equivalent (n=6 each) indicating respective per cent mean recovery as 103 ± 0.9, 101.3 ± 1.1 and 99.4 ± 1.5. There was no difference between the mean values by the comparable methods (P>0.1, n= 6 each), and overall estimated mean was found to be equivalent of 30.3 ± 0.2 µg of diosmin showing mean per cent recovery as 101 ± 0.8 in Daflon-500 tablets. This enabled treating Daflon-500 tablets as having 100 per cent purity.

Similarly, recovery of 30 µg diosmin added to 30 µg of daflon was estimated to be comparable with the three methods (**Table 4)** with no difference between mean recovery of diosmin (P>0.1, n= 6 each) and recovery remained about 98 per cent. The coefficient of variation ranged from 0.9 to 2.0.

Flavonoid content in purified orange peel extracts

Orange peel powder was chosen for determination of flavonoid content as it contains flavonoids such as diosmin, hesperidin, neohesperidin and naringin[27]. Estimated flavonoid concentration in orange peel extracts was in the range of about 6 to 8 µg per mg powder. Ethanolic extract showed nearly 18 % higher yield compared to methanolic extract (P<0.01). The respective mean values, 8.2 ± 0.2 and 6.7 ± 0.1 obtained by HCl method were not different from those obtained correspondingly with $AlCl_3$, 8.1 ± 0.1 and 6.5 ± 0.1 µg diosmin equivalent per mg powder (P>0.1) whereas those obtained correspondingly with $AlCl_3$ + HCl, 6.9 ± 0.1 and 5.7 ± 0.1 were nearly 15 % lower (P<0.01). This implied that the orange peel extracts suffered some loss in flavonoid, possibly due to its flavanone contents such as naringin that binds poorly with aluminium ions[3, 8], by additional processing required during $AlCl_3$ + HCl method. Preliminary experimentations using extracts from tobacco leaves and citrus fruit peel extracts (lemon, orange and grapefruit) have also confirmed that the acid method was applicable to other flavonoids.

Table 4: Recovery of added diosmin to daflon

Method	Total flavonoid recovered, µg[a]	Diosmin recovery Mass, µg	% Recovery
HCl	60.2 ± 0.4	29.3 ± 0.4	97.6 ± 1.3
$AlCl_3$	59.8 ± 0.4	29.4 ± 0.4	98.2 ± 3.0
$AlCl_3$ + HCl	58.3 ± 0.1	29.4 ± 0.1	97.9 ± 0.4

* A mixture of 60 µg flavonoid containing 30 µg each of daflon and disomin in 1 mL of 0.1 N NaOH. The values are mean ± s.e. of six observations each.

Table 5: Diosmin-like activity in purified orange peel extracts (µg $^{-1}$ mg)

Method	**PEOPE[a]**	**PMOPE[b]**
HCl	8.2 ± 0.2	6.7 ± 0.1
Aluminium chloride	8.1 ± 0.1	6.5 ± 0.1
Aluminium+ HCl	6.9 ± 0.1	5.7 ± 0.1

The values are mean ± s.e.. of 10 observations each.

[a] Purified ethanolic orange peel extract

[b] Purified methanolic orange peel extract

CONCLUSIONS

A simple, novel method for quantitative determination of flavonoids in test samples using concentrated HCl has been provided. The color formed is independent of phenolic residues and/or sugar component of flavonoids. The method has been tested with diosmin, daflon and orange peel extracts and provides comparable values to those provided by standard colorimetric general purpose method using aluminium chloride. An improvised method has also been devised to combine two techniques: using aluminium ions to complex with flavonoids followed by precipitation and purification of complex, and subsequent determination by HCl method. Extended studies have revealed applicability of acid method for assaying rutin, quercetin, morin and extracts from tobacco leaves and rinds of lemons and grape fruits (unpublished data).

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