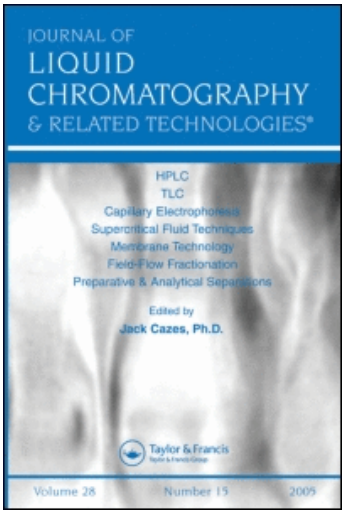

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SOLVENT SYSTEMS FOR THE COUNTER-CURRENT CHROMATOGRAPHY OF HYDRO-PHOBIC NEUROPEPTIDE ANALOGS AND HYDROPHILIC PROTEIN FRAGMENTS

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ABSTRACT

Two-phase solvent systems used in countercurrent chromatography conducted in Ito coil planet centrifuges have been found useful for fractionating solid-phase synthesized cholecystokinin and bombesin analogs. A system composed of chloroform, acetic acid and water has been found to be quite useful for these hydrophobic peptides which are not water soluble. On the other hand, common types of peptides synthesized are protein partial sequences produced for generating antibodies. The most hydrophilic sequences of proteins are usually selected for this purpose. The results of chromatographing a series of such peptides in the n-butanol, acetic acid and water system are presented.

INTRODUCTION

With our increasing experience in the synthesis and purification of many types of peptides, we have developed countercurrent chromatographic conditions suitable for the fractionation of small highly aromatic peptides such as the unsulfated cholecystokinin analogs <2> and a series of carboxyl-terminal partial sequences of bombesin. We also have prepared a series of

pentadecapeptides of varying composition that are hydrophilic sequences of proteins. These are to be conjugated to protein carriers for immunization in generating antibodies for biological studies. The composition without the sequence is given for these peptides which represents an opportunity to observe the correlation of aromatic groups in the structure to elution volume in a commonly used solvent system, n-butanol, acetic acid and water (4:1:5, by volume). Thus, conditions for two kinds of peptides are described here: small hydrophobic peptides and larger polar peptides. The instrument primarily used in these experiments is the horizontal flow-through coil planet centrifuge. In one example the multi-layer coil planet centrifuge achieved a rapid separation.

METHODS

The peptides were synthesized via solid-phase methods usually on 1 g of p-methyl-benzhydrylamine resin which gave the peptide amides after cleavage <3>. The Boc protected L-amino acids used included 2-Cl-CBZ-Lys, O-2-Br-CBZ-Tyr, B-Bzl-Asp, Bzl-Glu, O-Bzl-Ser, O-Bzl-Thr, Tos-Arg, Tos-His and in some syntheses Xan-Asn and Gln. Fmoc-D-Tyr and Fmoc-Gly were used in the synthesis of a CCK analog (Fig. 1). The Boc amino acids were coupled in 2.5 molar excess over the amino component with an equimolar amount of dicyclohexylcarbodiimide and hydroxybenzotriazole in methylene chloride and dimethylformamide for 2 hr. The coupling was monitored by the qualitative ninhydrin reaction on a sample of the resin. The coupling was repeated until a negative ninhydrin test. The Boc group was deprotected by 25% trifluoroacetic acid in methylene chloride and indole 1 mg/ml. The peptide-resin was washed and neutralized by 10% triethylamine

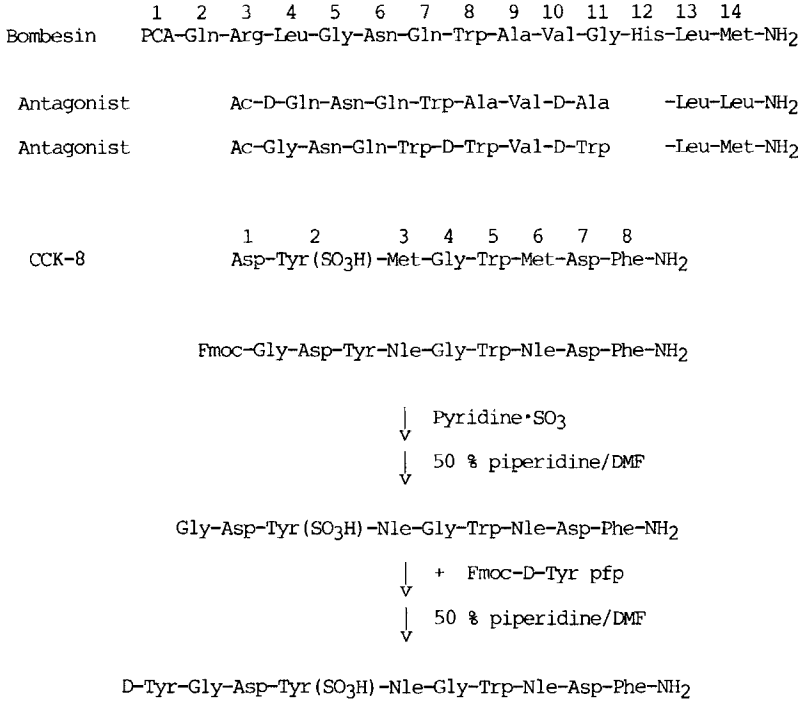

FIGURE 1

Structures of the synthetic neuropeptide analogs purified by CCC. Above are deletion analogs missing His for antagonist activity. The Fmoc-Gly-CCK-8 peptide purified by CCC is shown below the structure of CCK-8. The peptide was used in the preparation as shown of an analog with the D-Tyr at the amino end that could be selectively radioiodinated by the chloramine T method to give a biologically active radiolabeled ligand for the CCK receptor.

in methylene chloride. After the repetition of these steps until the desired sequence was completed, the peptide-resin was treated with hydrogen fluoride at 0°C for 45 min in the presence of anisole, 1 ml/g resin, which removed peptide from the resin and the side chain protecting groups. Some syntheses were conducted in a Biosearch Sam Two peptide synthesizer (San Rafael, CA) following the above protocol. After the cleavage the resin was washed with ether or ethyl acetate, dried and finally extracted with glacial acetic acid in the case of the hydrophobic peptides or water for the pentadecapeptides. The extract was lyophilized to yield a powder that could be directly loaded onto the coil planet centrifuge.

Most of the separations were performed in the horizontal flow-through coil planet centrifuge described previously <2,4,5>. Two prototype instruments, one built by the Laboratory of Technical Development, National Heart, Lung and Blood Institute, National Institutes of Health (Bethesda, MD) and the other by Kontes Scientific Glassware/Instruments (Vineland, NJ) were used for this work. The column-coil is comprised of 2.6 mm i.d. PTFE tubing holding a volumne of 285 ml. For the purification of a CCK analog the multi-layer coil planet centrifuge available from P.C. Inc. (Potomac, MD) with 1.6 mm. i.d. tubing holding approximately 320 ml of liquid was utilized <6,7>. Milton Roy and LDC mini-pumps (Sunnyvale, CA) provided solvent delivery at either 24 ml/hr or 300 ml/hr and fractions of 6 ml were collected in a LKB Ultro-rac fraction collector (Gaithersburg, MD). Usually the absorbance of the fractions were read manually and peak tubes were combined, flash evaporated and lyophilized in a small volume of glacial acetic acid or water. The peptides were analyzed for homogeneity by HPLC, TLC and amino acid analysis.

RESULTS AND DISCUSSION

Substitutions and deletions were made in the minimally active sequence of bombesin (positions 5 to 14, Table 1) for biological testing as agonists or antagonists in receptor mediated activity and in feeding (data reported elsewhere). All of the peptides were purified by countercurrent chromatography. Examples of the separation and analysis of two peptides are described here. In Fig. 2 is shown the separation of 95 mg of synthetic [N-Ac, D-Gln^{7}, D-Ala^{11}, Leu^{14}, des His^{12}]BBN(7-14), an

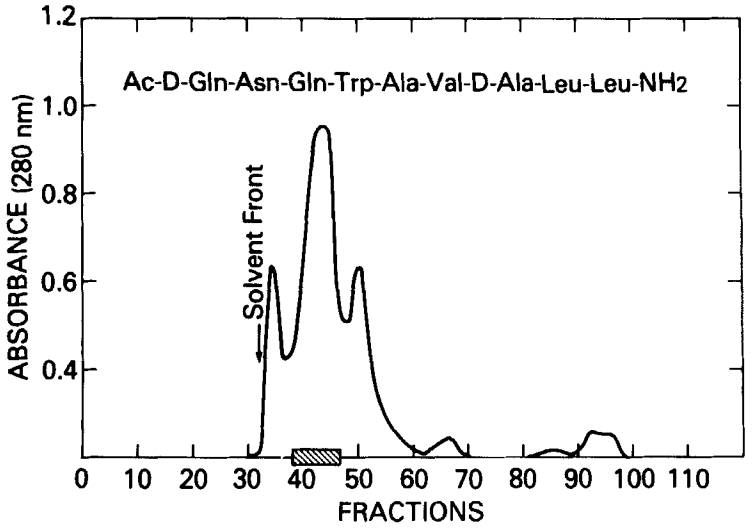

FIGURE 2

Countercurrent chromatography of the bombesin antagonist analog, [N-Ac, D-Gln^{7}, D-Ala^{11}, Leu^{14}, des His^{12}]BBN(7-14) in the flow-through coil planet centrifuge. A sample of 95 was chromatographed with the upper or aqueous phase of chloroform, acetic acid and water (2:2:1) run as the mobile phase at 24 ml/hr at a rotation rate of 400 rpm. Fractions of 15 min or 6 ml were collected. Pure peptide, 33 mg, was recovered from fractions 38-47.

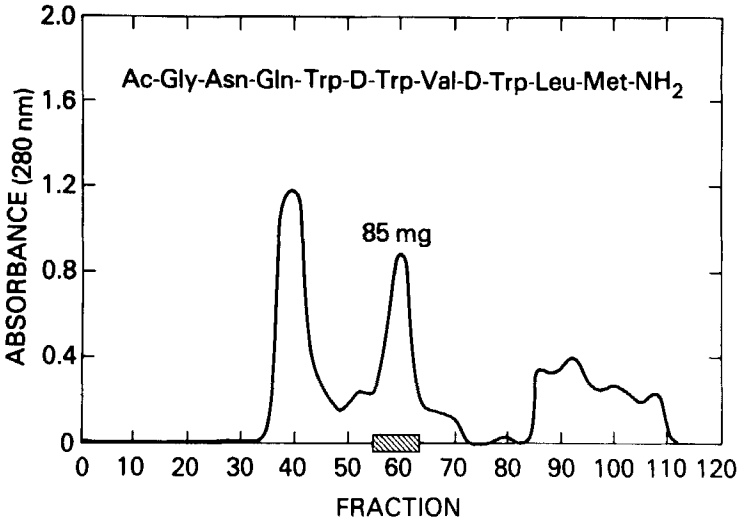

FIGURE 3

Chromatography of the bombesin antagonist, [N-Ac, D-Trp^{9},D-Trp^{11}, des His^{12}]BBN(5-14) in the flow-through coil planet centrifuge in the same solvent and conditions described in Fig. 2. A sample of 400 mg was loaded and 85 mg pure peptide was recovered.

antagonist analog, carried out in the upper or aqueous phase of the chloroform/acetic acid/water system. The tubes comprising the peaks were concentrated and analyzed by HPLC and TLC. The recovery of pure peptide from the major peak (38-47) was 33 mg.

Another antagonist analog [Ac,D-Trp^{9},D-Trp^{11}, des His^{12}]BBN (5-14) was synthesized and 400 mg was chromatographed in the same chloroform solvent system (Fig. 3). There was material at the solvent front and a retained peak. The HPLC analysis of the contents of both peaks (Fig. 4) showed fraction 55-63 to be pure. Amino acid analysis showed the presence of the expected amino acid residues. The order of elution of the contents of both fractions in reversed phase chromatography corresponded to

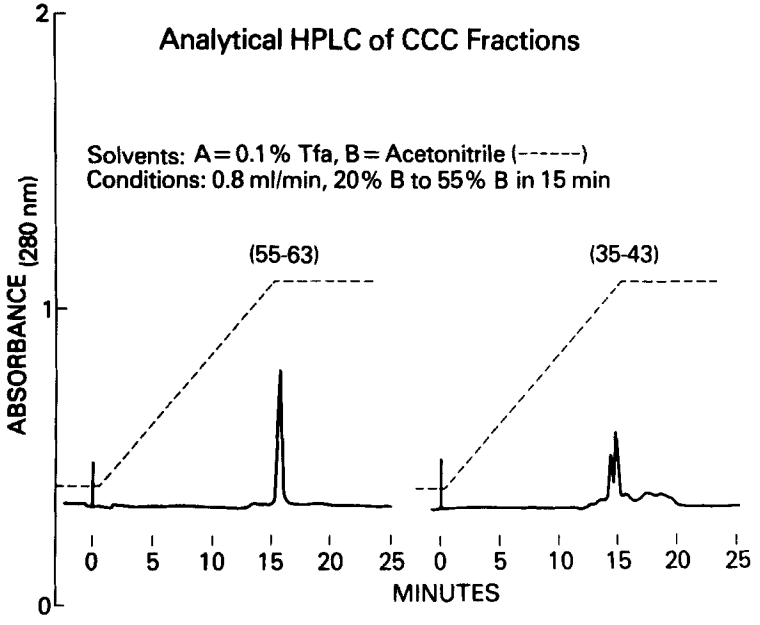

FIGURE 4

High performance liquid chromatographic analysis of the contents of the solvent front fractions 35-43 and retained peak 55-63 of the countercurrent chromatography of Fig. 3. A sample of 50 ug was chromatographed on a uBondapak C_{18} column (.4 X 15 cm) in the conditions given in the figure. Detection was at 280 nm at 2 absorbance units full scale.

the order of elution in countercurrent chromatography. The more polar material that was eluted near the solvent front of the aqueous mobile phase was eluted earlier in HPLC.

For the preparation of D-Tyr-Gly-Asp-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 used as a radioiodinated ligand for cholecystokinin binding studies (R.T.Jensen, unpublished results and 5), Fmoc-Gly-Asp-Tyr-Nle-Gly-Trp-Nle-AspPhe-NH_2 was first synthesized, then chromatographed in the conditions described here. Afterwards, the peptide was sulfated and the Fmoc group was

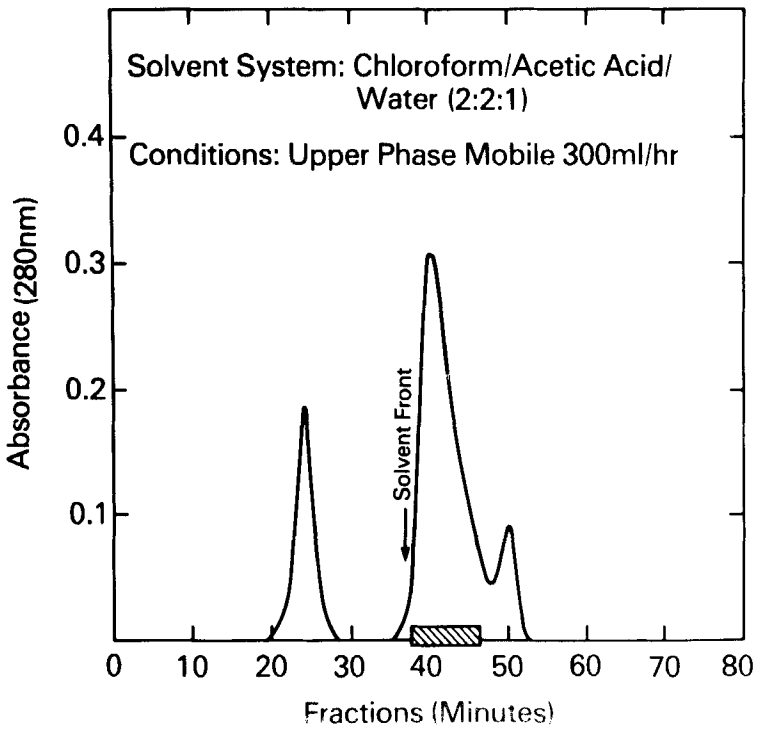

FIGURE 5

Chromatography of Fmoc-Gly-unsulfated CCK-8 on the multi-layer coil planet centrifuge. The chloroform, acetic acid, water system with the upper phase mobile was used at a flow of 300 ml/hr and rotation rate of 800 rpm. A sample of 100 mg was loaded and 6 ml fractions collected. The contents of the major peak was the pure peptide.

removed (Fig. 1). The product was purified and coupled in solution to Fmoc D-Tyr pentafluorophenyl ester and finally deprotected again and chromatographed to afford the D-Tyr CCK analog. The initial solid-phase synthetic product was chromatographed on the multi-layer coil planet centrifuge in the chloroform/acetic acid/water solvent system (Fig. 5). A sample of 100 mg was loaded and the aqueous mobile phase was eluted from

the external tail end of the coil to the internal head direction at 300 ml/hr and a centrifugal rate of 800 rpm. The major peak contained 67 mg of the pure peptide and a total of 82 mg of material was recovered (Fig. 5). As indicated in the figure, the separation was complete in 1 hr. This solvent at room temperature makes rapid countercurrent chromatography possible. All of the foregoing peptides were not water soluble and had to be dissolved in glacial acetic acid for lyophilization or chromatography by HPLC. The peptides were soluble in the two-phase solvent system used in these experiments.

In the course of purifying pentadecapeptides that are hydrophilic sequences of proteins to be used as antigens in antibody formation we used the more polar solvent system, n-butanol, acetic acid and water (4:1:5). In Table 1 are the compositions of the peptides and their behavior in this solvent system as indicated by their peak fractions and their partition coefficients calculated from the run <2>. They are grouped in the order of elution using the upper phase mobile. For peptides bearing multiple aromatic residues that were expected to elute with the solvent front in a mobile upper phase, the lower phase was used as the mobile phase to retain the sample and afford better separations of side products (last two peptides in the table). The results do not show gradual fractionation of all the peptides in the solvent system but the peptides that were eluted in a mobile upper phase with a K of at least 1 had to have 1 aromatic group if the overall charge was negative or 2 aromatic groups if the charge was positive. The peptides that had very low Ks were more positively charged with variable aromaticity. In this solvent system positively charged peptides are more polar. A peptide with one aromatic group that

Table 1

Chromatography of Pentadecapeptides in the Flow-through Coil Planet Centrifuge in n-butanol, acetic acid and water (4:1:5)

Composition	Charge*	No. Aromatic Groups	Mobile Phase	Peak Tube	K**
Ala.Arg.Asn.Asp.Gly.Ile. 2Leu.2Phe.Ser.4Thr.	+2	2	U (upper)	33	2.2
Ala.2Asn.3Asp.Gly.3Leu. 2Pro.Ser.Thr.Tyr.	+1	1	U	43	1.2
Arg.Cys.Gly.His.4Leu.2Met. 3Ser.Tyr.Val.	+3	2	U	47	1.1
Ala.2Asn.Asp.Glu.Gln.Ile. Leu.Lys.Met.Ser.4Thr.	+2	0	U	115	<.2
Asn.Gln.His.Ile.Leu.3Lys. 3Met.Ser.3Thr.	+5	1	U	129	<.2
Ala.2Arg.2Asn.Cys.Glu.His. 2Ile.Leu.Met.2Ser.Thr.Tyr.	+4	2	U	149	<.2
3Ala.Cys.3Glu.Gly.Ile.Met. Phe.Pro.2Thr.2Tyr.	+1	3	L (lower)	45	1.1
Cys.2Ile.Leu.2Met.4Phe. Pro.Ser.3Thr.	+1	4	L	117	<.2

* Charge = Relative charge of peptide in acetic acid solution.

** K = concentration in mobile phase/conc. in stationary phase, calculated from the elution volume (2).

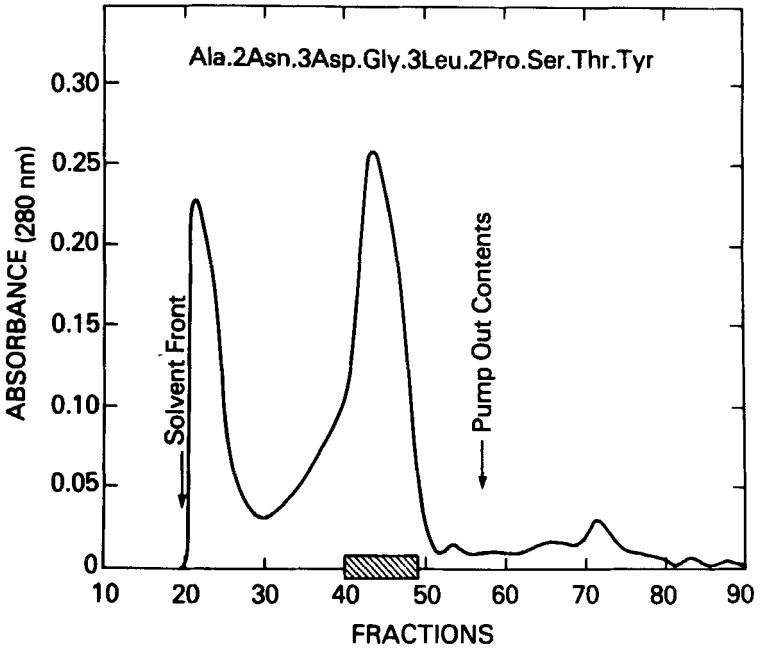

FIGURE 6

Chromatography of 1 g of a pentadecapeptide containing one aromatic group in the flow-through coil planet centrifuge in the solvent system, n-butanol, acetic acid and water (4:1:5) with the upper phase mobile at 24 ml/hr and a rotation rate of 400 rpm. Fractions 40-49 contained purified peptide.

was negatively charged had a high partition coefficient (Fig. 6) eluting after some retention and a nonaromatic neutral peptide had a very low partition coefficient (Fig.7). In the table the net charge of each peptide is listed relative to the pH of the acetic acid solution. The order of elution is inversely proportional to net positive charge and is affected by hydrophobic aromatic groups. From this work it is evident that a solvent

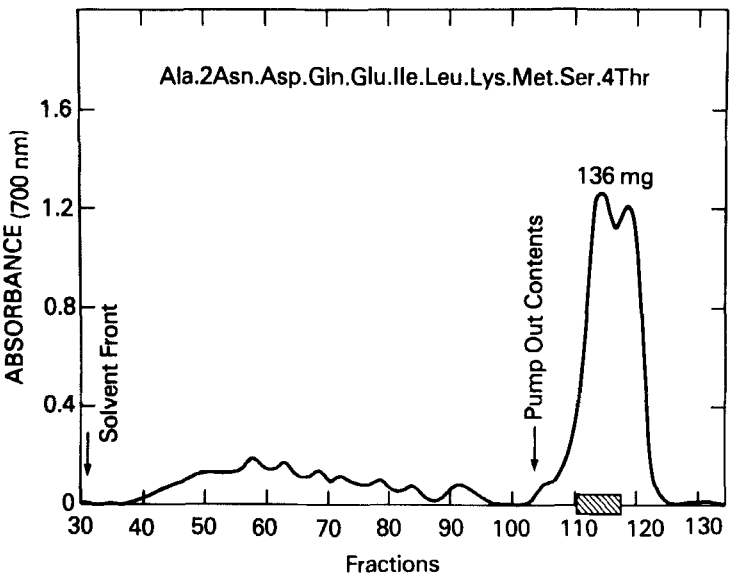

FIGURE 7

Chromatography of a nonaromatic pentadecapeptide in the same conditions as described in Fig. 6. The purified peptide was contained in fractions 110 to 118. The second peak did not have the expected molar ratios of amino acids. Detection of peptide was by Folin-Lowry reaction.

system capable of fractionating cationic peptides is necessary to be developed. Experience is being acquired with peptides of varying sizes and composition. Correlation of their partition coefficients with a hydropathic index, for example, will serve in the prediction of behavior among a choice of solvent systems.

ACKNOWLEDGEMENTS

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1. Abbreviations used: CCC = countercurrent chromatography; HPLC = high performance liquid chromatography; TLC = thin layer chromatography; Boc = butyloxycarbonyl; Tos = tosyl; CBZ = carbobenzoxy; Bzl = benzyl; Fmoc = fluorenylmethyloxycarbonyl; Xan = xanthydryl; PTFE = polytetrafluoroethylene; CCK = cholecystokinin; BBN = bombesin; DMF = dimethylformamide.

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