LC/MSを用いた健康食品中のヒドロキシチオホモシルデナフィル，アミノタダラフィル，チオシルデナフィル，ジメチルシルデナフィル，チオジメチルシルデナフィルの一斉分析

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Report

Simultaneous Identification of Hydroxythiohomosildenafil, Aminotadalafil, Thiosildenafil, Dimethylsildenafil, and Thiodimethylsildenafil in Dietary Supplements Using High-Performance Liquid Chromatography-Mass Spectrometry

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We developed a method for the separation and identification of illegal adulterants (hydroxythiohomosildenafil, aminotadalafil, thiosildenafil, dimethylsildenafil, and thiodimethylsildenafil) from dietary supplements using high-performance liquid chromatography-mass spectrometry. The separation was achieved on a C_{18} column: the mobile phase consisted of 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v) and acetonitrile, with gradient elution at a flow rate of 0.2 mL/min. The proposed method could also be used to separate vardenafil, homosildenafil, and dimethylsildenafil, all of which have the same molecular weight. Furthermore, the proposed method could simultaneously separate hydroxythiohomosildenafil, aminotadalafil, thiosildenafil, dimethylsildenafil, thiodimethylsildenafil, vardenafil, and homosildenafil. Thus, this method may be useful to identify medicinal ingredients for erectile dysfunction and their analogs and to control the quality of dietary supplements.

Key words: LC-MS; dietary supplement; erectile dysfunction; medicinal ingredients

Introduction

In recent years, adverse effects of dietary supplements that contain medicinal ingredients has become a social problem[1]. To prevent impairment of health by medicinal ingredients present in dietary supplements, it is necessary to analyze the components commercial of dietary supplements. Inhibitors of phosphodiesterase type 5 (PDE-5) such as sildenafil, tadalafil, and vardenafil are administered to treat erectile dysfunction and have recently been detected in dietary supplements[2),3)]. The known side effects of PDE-5 inhibitors include headaches and visual abnormality. Mortality could also result from the combined use of PDE-5 inhibitors and nitric monoxide donors[1]. Various ingredients whose structures have been modified from those of medicinal ingredients, such as sildenafil, in order to avoid identification have also been detected in dietary supplements[4)–7)]. Sometimes, more than one kind of medicinal ingredient or analog is detected in a single dietary supplement[8]. For example, in 2011, we detected 5 analogs (hydroxythiohomosildenafil (HT; 13 mg/capsule), aminotadalafil (AT; 10 mg/capsule), thiosildenafil (TS; 5 mg/capsule), dimethylsildenafil (DS; 53 mg/capsule), and thiodimethylsildenafil (TDS; 10 mg/capsule)) in a single dietary supplement[8].

Generally, medicinal ingredients and analogs are quantified by HPLC-UV and identified by LC-MS. It is critically important to separate the peaks of multiple medicinal ingredients and analogs in order to accurately quantify (by HPLC-UV) and identify (by LC-MS) them. It is also undesirable to use counter ions for the LC-MS mobile phase. In this study, we developed a method to separate 5 analogs using LC-MS.

Materials and Methods

Chemicals and reagents

LC-MS grade acetonitrile, methanol, and formic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). LC-MS grade ammonium formate, aqueous ammonia solution, and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HT, DS, and TDS standards were purchased from TLC Pharma Chem (Ontario, Canada). The TS standard was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The AT standard was purchased

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from Toronto Research Chemicals (Ontario, Canada). The vardenafil standard was provided by the National Institute of Health Sciences in Japan. Standard stock solution of homosildenafil (75.5 ppm) was provided by the Tokyo Metropolitan Institute of Public Health, Department of Pharmaceutical Sciences.

Instrumentation and chromatographic conditions

The LC-electrospray ionization-MS experiments were performed using a Prominence UFLC liquid chromatography system and an LCMS-2020 quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). An Inertsil ODS-3 column (5 mm particle size, 2.1 mm i.d.×150 mm) (GL Science, Tokyo, Japan) was used. The following gradient system was used with mobile phase A (5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v)) and mobile phase B (acetonitrile) delivered at 0.2 mL/min; A : B, 100 : 0 (0–3 min); 70 : 30 (13–20 min); and 50 : 50 (30–50 min). The injection volume was 1 mL. The column temperature was 40℃. The instrument parameters were as follows: source temperature, 350℃; desolvation temperature, 250℃; and desolvation gas flow, 600 L/hr. The mass range of the spectra was *m/z* 100–800 in the scan mode analysis. All LC/MS was performed in both positive and negative modes.

Preparation of standard solutions

Each standard was dissolved in methanol to make 100–500 μg/mL standard stock solutions. The working standard solutions (1 μg/mL) were then prepared from the standard stock solutions.

Sample preparation

The dietary supplement (10 mg) investigated was either a powdered tablet or the contents of a capsule. Spiking solution (each medicinal ingredient: 10 μg) was added. After 30 min, the sample was extracted with 10 mL of methanol by sonicating for 10 min. The solution was then filtered through a 0.45-μm pore size membrane prior to LC-MS analysis.

Results and Discussion

Optimization of chromatographic separation conditions

Except for the mobile phase A and the flow rate, chromatographic conditions were based on the methods of the National Institute of Health Sciences in Japan[5]. The method has previously been used to analyze 20 different medicinal ingredients and analogs using 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v) as mobile phase A. Though this method is very useful, it does not measure HT, DS and TDS. Initially, 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v) was used as mobile phase A to analyze the standard solutions of all 5 analogs (HT, AT, TS, DS, and TDS, each at a concentration of 1 μg/mL). To plot the mass chromatogram, *m/z* 521 (HT), 491 (TS), 489 (DS), and 505 (TDS) in the positive mode and 389 (AT) in the negative mode were extracted as major peaks of the 5 analogs. Our results showed that HT and TDS were not separated. This was expected, since previous reports have shown that the retention time of TDS is very close to those of TS and HT in HPLC analysis[9]. Takahashi *et al.* achieved separation of TDS and DS using HPLC/UV and 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (70 : 30, v/v) as a mobile phase under isocratic conditions[10]. We therefore attempted to use alkaline conditions to separate the 5 analogs. When 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (75 : 25, v/v) was used as the mobile phase A, the chromatogram pattern changed markedly. But, although HT and TDS were separated, HT and DS were not.

To improve the separation of the 5 analogs, we investigated the retention time of each analog when 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v), 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v), 10 mM ammonium bicarbonate (pH 7.9)–acetonitrile (75 : 25, v/v), 10 mM ammonium bicarbonate (pH 9.0)–acetonitrile (75 : 25, v/v), and 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (75 : 25, v/v) were used as mobile phase A (Fig. 1). We found that the 5 analogs were separated when 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v) was used as mobile phase A.

Detection limit

The molecular ions ($[M+H]^+$, $[M-H]^-$) were the major peaks for each compound in both the positive and negative modes. The detection limit of each compound was estimated based on a signal-to-noise ratio of 3. The detection limits were 50 ppb (TS), 250 ppb (AT), 10 ppb (TDS), 25 ppb (DS), and 75 ppb (HT). To plot the mass chromatogram, *m/z* 521 (HT), 491 (TS), 489 (DS), and

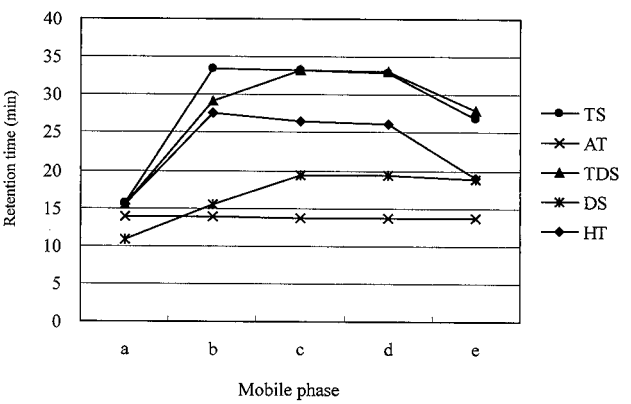

Fig. 1. Retention times of the 5 analogs using 5 different kinds of mobile phase A. The concentration of all compounds was 1 μg/mL

(a) 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v)
(b) 5 mM ammonium formate (pH 6.3)–acetonitrile (75 : 25, v/v)
(c) 10 mM ammonium bicarbonate (pH 7.9)–acetonitrile (75 : 25, v/v)
(d) 10 mM ammonium bicarbonate (pH 9.0)–acetonitrile (75 : 25, v/v)
(e) 10 mM ammonium bicarbonate (pH 10.0)–acetonitrile (75 : 25, v/v)

505 (TDS) in the positive mode and *m/z* 389 (AT) in the negative mode were selected. In terms of sample preparation, the detection limits of the 5 analogs were below 250 μg/g. Thus, the proposed method has sufficient sensitivity to detect medicinal ingredients, since the prescribed dosage of sildenafil, tadalafil, and vardenafil is over 5 mg.

Recovery and precision

The proposed method was evaluated using the standard addition method to 2 capsules and 1 tablet, which did not contain the 5 analogs. Recovery rates of the 5 analogs when standards were spiked at 10 μg/10 mg were between 66.7% and 146.3%, and the relative standard deviations (RSD) were between 1.7% and 29.7% (Table 1).

The recovery rates and RSD were not satisfactory to quantify the amounts of the 5 analogs. Generally, medicinal ingredients and analogs are quantified by HPLC-UV. LC-MS is usually used to identify compounds, and not to quantify them. Therefore, the recovery rates and RSD were satisfactory for the identification of the 5 analogs.

Table 1. Recovery of 5 analogs from 3 dietary supplements

Compound	Capsule 1		Capsule 2		Tablet 1	
	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
TS	84.8	11.2	83.7	12.9	72.4	17.2
AT	66.7	17.6	146.3	29.7	109.5	21.3
TDS	92.8	1.7	93.6	6.0	79.2	3.4
DS	100.9	3.0	98.6	3.2	100.4	3.9
HT	101.8	2.5	91.9	3.4	76.8	1.9

($n = 3$)

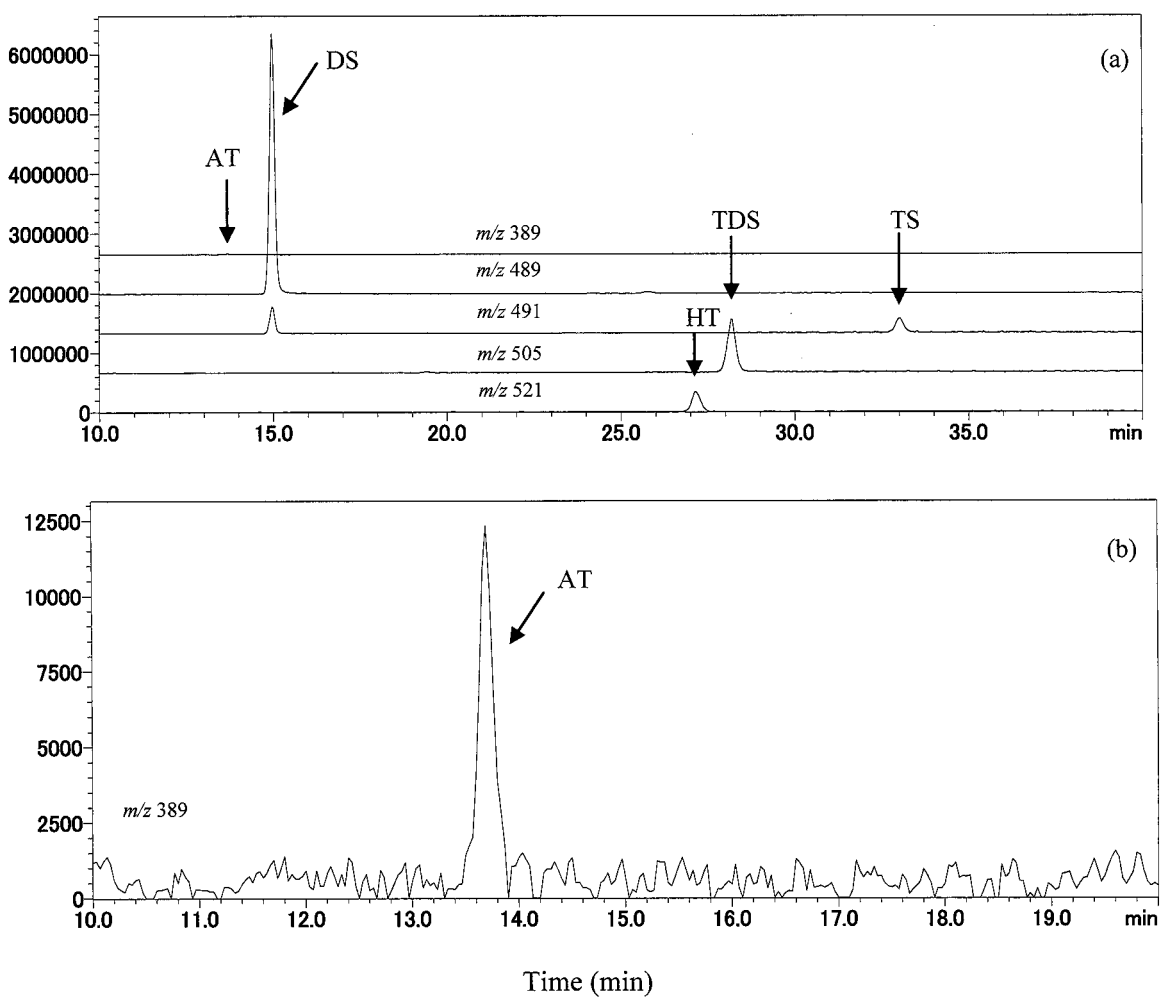

Fig. 2. Extract ion chromatograms of a dietary supplement containing all 5 analogs
a) *m/z*: 389,489, 491, 505, 521; (b) *m/z*: 389

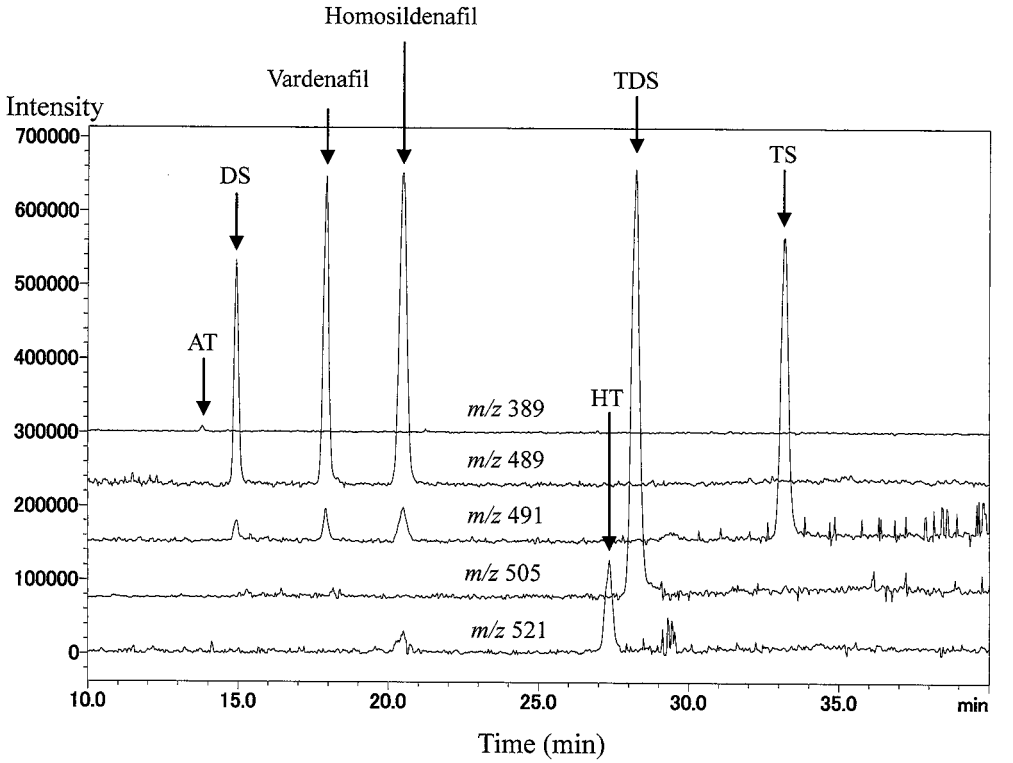

Fig. 3. Extract ion chromatograms of 7 compounds using the proposed method

The concentration of all compounds was 1 μg/mL

Application of the proposed method

We then attempted to apply the proposed method to a dietary supplement containing all 5 analogs. The sample solution was diluted 10-fold with methanol prior to LC-MS. The peaks of all 5 analogs were separated and clearly observed (Fig. 2).

It is very important to separate the peaks of medicinal ingredients and analogs, especially when a dietary supplement contains compounds possessing the same molecular weight. Vardenafil, homosildenafil, and DS have the same molecular weight, and the molecular ion (*m/z* 489) was the major peak for each compound in the positive mode. Standard solutions (each compound: 1 μg/mL) of the 3 compounds were therefore analyzed using the proposed method. The compounds were clearly separated (data not shown). Two peaks of homosildenafil and DS were not separated when 5 mM ammonium formate (pH 3.5)–acetonitrile (75 : 25, v/v) was used as mobile phase A (data not shown). Therefore, the proposed method is also useful for distinguishing vardenafil, homosildenafil, and DS. A standard solution of 7 compounds (HT, AT, TS, DS, TDS, vardenafil, and homosildenafil; concentration of each compound, 1 μg/mL) was separated into 7 peaks using our proposed method (Fig. 3). Thus, the proposed method could identify at least 7 compounds simultaneously suggesting that it may be applicable in quality control procedures of dietary supplements.

Conclusion

In this study, different LC-MS separation conditions were tested to develop a method for the separation of multiple medicinal ingredients and/or their analogs. The method developed could simultaneously identify HT, TS, DS, TDS, and AT in dietary supplements. The proposed method could also distinguish vardenafil, homosildenafil, and DS, all of which have the same molecular weight. Furthermore, HT, AT, TS, DS, TDS, vardenafil, and homosildenafil could be analyzed simultaneously. The proposed method may be useful for the separation and identification of medicinal ingredients and analogs and for quality control of dietary supplements.

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異なるタイプのステンレス表面におけるサルモネラ・エンテリティディスの生残とふき取り法による菌の回収（ノート，英文）

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食衛誌 **54**(3), 219～223 (2013)

組成と表面仕上げの異なる4種類のステンレス表面に添加した *Salmonella* Enteritidis の生残性と菌の回収について調査した．バイオフィルム産生能の異なる2株の *S.* Enteritidis をトリプトソイブロス（TSB）と卵黄液（EY）で培養し，ステンレスの表面に添加した．乾燥状態（22℃）で保管した後，綿棒を用いたふき取り法により生残菌を回収し，回収菌数とステンレス表面に残った菌数を測定した．バイオフィルム産生能の高い菌の生残率は，産生能の低い菌の生残率より高かった．TSB で培養添加された菌の生残率は，EY で培養添加された菌の生残率より高い傾向が見られた．ステンレス組成と表面仕上げの違いに基づく菌の生残率と回収率には有意の差は見られなかった．一例を除いて，添加された *S.* Enteritidis の98%以上が綿棒を使用したふき取り法により回収された．

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LC/MS を用いた健康食品中のヒドロキシチオホモシルデナフィル，アミノタダラフィル，チオシルデナフィル，ジメチルシルデナフィル，チオジメチルシルデナフィルの一斉分析（調査・資料，英文）

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土井崇広　梶村計志　沢辺善之

食衛誌 **54**(3), 232～236 (2013)

強壮効果を標榜したいわゆる健康食品からは，医薬品成分が検出された事例がある．また，近年では摘発を逃れるために，強壮効果のある医薬品成分の構造の一部を変えた医薬品成分類似体が検出される事例がしばしば見受けられる．大阪府では，いわゆる健康食品を対象とした試買調査を行っているが，平成23年度における調査において，1つの製品に，医薬品成分類似体5成分が配合されるという事例があった．LC/MS を用いて各成分を同定するためには，各成分を分離する必要があるが，ギ酸アンモニウム/アセトニトリルを用いた酸性条件における分析法では，一部の成分が分離せず，同定が困難であった．今回検討した分析条件は，医薬品成分類似体5成分を分離して同定することが可能であった．また，同じ分子量を有することから，同定が難しいバルデナフィル，ホモシルデナフィル，ジメチルシルデナフィルを明確に分離することも可能であった．以上のことから，今回検討した分析法は，いわゆる健康食品中の医薬品成分の同定法の1つとして有用であると考える．

＊大阪府立公衆衛生研究所

輸入茶中の残留農薬実態（1992年4月～2010年3月）（調査・資料）

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食衛誌 **54**(3), 224～231 (2013)

1992年4月から2010年3月にかけて東京都内で市販されていた輸入茶116検体について農薬の残留調査を行った．その結果，76検体から22種類の有機リン系農薬，有機塩素系農薬，ピレスロイド系農薬などが痕跡値（0.01 ppm 未満）～4.0 ppm の範囲で検出された．検出率は，不醗酵茶90%，半醗酵茶89%，醗酵茶49%と醗酵茶で低く，また，菌を使った発酵を行うプーアル茶では，有機リン系農薬は検出されなかった．発酵工程により農薬は減少するが，化学的に安定な有機塩素系農薬やピレスロイド系農薬は減少しにくく，検出されやすいことが示唆された．農薬が検出された輸入茶を喫食した場合の農薬の推定摂取量を算出し，一日摂取許容量（ADI）と比較したところ，エチオン45%を除いて各農薬 ADI 値の1%未満であり，通常の飲食による健康影響はないものと考えられた．

＊東京都健康安全研究センター

LC-MS/MS を用いた迅速な野菜類および果実類中の残留農薬一斉分析法の妥当性評価（妥当性評価）

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食衛誌 **54**(3), 237～249 (2013)

独自に開発した残留農薬一斉分析法について，「食品中に残留する農薬等に関する試験法の妥当性評価ガイドライン」（以下，ガイドライン）に則って評価を行った．本法は，ポリプロピレン製遠心管に採取した試料をアセトニトリルで抽出後，塩化ナトリウム，無水硫酸マグネシウムおよびクエン酸塩を添加して塩析・脱水し，得られたアセトニトリル相をグラファイトカーボン/PSA 積層カラム（かんきつ類では C18 カラムを追加する）で精製し，LC-MS/MS で分析する．本法を8種類の野菜類および果実類に適用した．各食品に 0.01 および 0.05 μg/g になるように161種類の農薬を添加して，各濃度において分析者1名が併行数2で5日間の枝分かれ試験を行い，真度，併行および室内精度を算出した．その結果，両濃度で8種類すべての野菜類および果実類についてガイドラインに示される目標値を満たした農薬は127種類であった．

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