Using Dried Blood Spots in Combination with UHPLC and Enhanced Ion Generation ESI to Streamline Pharmacokinetic Assays

Application Note

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Abstract

This application note demonstrates the productivity performance of the Agilent 1290 Infinity LC coupled to the Agilent 6460A Triple Quadrupole LC/MS.

The following will be shown:

- Use of dried blood spot (DBS) technology to extract a cassette of pharmaceuticals from merely 15 µL of full blood
- High speed chromatography / MS at column back pressures approaching 1,200 bar using a novel ZORBAX Rapid Resolution High-Definition (RRHD) sub-2 µm particle column
- High quality separation of a cocktail of seven small molecule drugs in less than 50 sec
- High-end detection sensitivity

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Introduction

The use of LC/MS/MS for quantitative bioanalytical measurements may be considered the hallmark of pharmacokinetic (PK) screenings. Preclinical *in vivo* screens involve the administration of a lead compound to a number of animals (typically mice or rats) whose plasma is consequently monitored over the course of time for drug absorption and decay. Nowadays, PK evaluation is conducted early in drug discovery to prioritize and proceed with new chemical entities (NCE) that have the best chance to pass the later stages in drug development. However, large numbers of NCE's, combined with the pressure to quickly and cheaply fail non-drug-like compounds early, require fast sample turnaround.[1] The term cassette dosing (a.k.a. "cocktail" or "N-in-one") refers to an approach that is widely used to accelerate PK screenings. This entails the simultaneous administration of several drugs to the single laboratory animal. The parallel testing leads to fewer rodents, less animal handling, sample preparation and reduced analysis. This complements ethical arguments and has considerable cost benefits.[1-4]

This work presents the LC/MS/MS bioanalysis of a cassette containing six pharmaceuticals. Prime objectives were to demonstrate maximum productivity without sacrificing chromatographic resolution and/or sensitivity. An Agilent 1290 Infinity LC with a short RRHD column (50 x 2.1 mm ID, 1.8 µm) was used to allow flow rates up to 2 mL/min at a column back pressure of 1,200 bar.[5] The LC System was coupled to a 6460A Triple Quadrupole LC/MS for two reasons: high-end sensitivity and the system's capability to handle fast flow rates without the necessity to split. The high flow rates can be accommodated by the Agilent Jet Stream technology.[6]

Dried Blood Spot (DBS) technology[7-10] was used to extract the cassette from small volumes of whole blood. DBS offers advantages over traditional plasma sampling for pre-clinical and clinical assays. For example, in pre-clinical laboratories, the reduced volumes of blood needed to prepare DBS samples allows serial bleeding of a reduced number of rodents, which aids overcoming inter-individual variations. This could improve the quality of PK data in addition to the related ethical and cost benefits.

Experimental

Reagents

All standard chemicals [dextromethorphan, verapamil, methoxyverapamil, imipramine, amitriptyline, protriptyline and trimipramine (ISTD), formic acid (FA), trifluoroacetic acid (TFA)] were from Sigma-Aldrich (Germany). HPLC grade water was from Burdick & Jackson (USA). HPLC grade acetonitrile (ACN) and methanol (MeOH) were from Merck (Germany).

Samples

Approximately 2 mg/mL solutions of each compound were prepared in ACN/water 50:50 v/v. These seven stock-I solutions were mixed to give equal concentrations (100 µg/mL) of each of the six test drugs in a stock-II mix. Stock-II mix was then diluted to give a set of reference standards to obtain a blank and concentrations in the range 0.5 to 5,000 ng/mL. 10 µL of each reference standard was spiked into 90 µL of rat blood to give a blood blank and blood standards in the range 0.05 to 500 ng/mL.

DBS extraction procedure

FTA Elute MicroCards (FTA-cards) were from Whatman (UK). **Fig. 1** shows 15 µL aliquots of blood standards spotted (left). Disks of 6 mm ID were punched from the dried blood spot using a Harris Uni-Core puncher (Sigma-Aldrich, Germany). Fig. 1 shows that a rim of blood remained after punching (right). Disks were placed into a 2 mL Eppendorf tube in which 100 µL of ACN/water 60:40 v/v [2% FA] was added. Extraction was achieved by vortex-mixing for a few seconds before ultra sonication for 15 min. After centrifugation at 15,000 rpm, the blood-extracts were transferred into clean vials. In order to simplify sample preparation, the 60% ACN-extracts were injected directly onto the LC/MS system without reconstitution or dilutions.

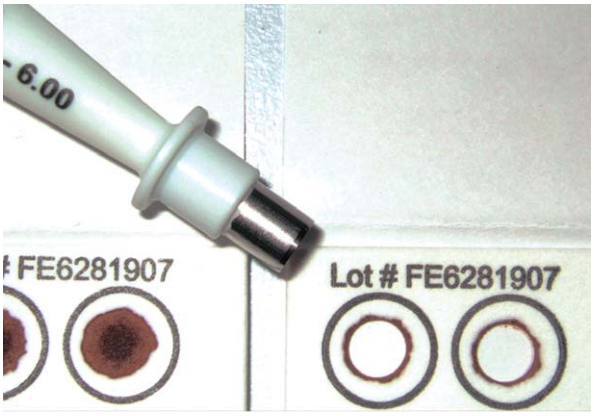

Fig.1: Picture of FTA-cards and the 6 mm ID puncher. Left: A 15 µL blood spot. Right: Spots in which a 6 mm ID disk was punched.

Equipment

- Agilent 1290 Infinity LC System comprising of 1290 Infinity Binary Pump with integrated degasser, 1290 Infinity Autosampler with thermostat and 1290 Infinity Thermostatted Column Compartment
- Agilent 6460A Triple Quadrupole LC/MS System with Agilent Jet Stream technology
- Agilent MassHunter Workstation software for instrument control, data acquisition and data processing
- Agilent MassHunter Optimizer software
- Agilent Rapid Resolution High Definition (RRHD) ZORBAX Eclipse Plus - C18, 2.1 x 50 mm, 1.8 µm column

Agilent 1290 Infinity Method

Solvent A:	Water [0.1 % FA]	
Solvent B:	MeOH [0.1 % FA]	
Gradient:	t (min)	% B
	0	40
	1	60
	1.2	90
	1.7	90
	1.9	40
Flow rate:	1 mL/min	
T (column):	50°C	
Stop time:	2 min	
Post time:	1 min	
Injection volume:	3 µL	
Needle wash:	15 sec with ACN/water 60/40 v/v (0.1% TFA)	

Agilent 6460A Triple Quadrupole LC/MS Conditions

Scan type: MRM (MassHunter Optimizer software used to obtain settings given in **Table 1**

Ionization mode: ESI positive with Agilent Jet Stream technology

Drying gas temperature:	300°C
Drying gas flow:	11 L/min
Nebulizer pressure:	35 psig
Capillary voltage:	2,250 V
Sheath gas temperature:	400°C
Sheath gas flow:	12 L/min
Nozzle voltage:	0 V

Compound	ISTD	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Quantifier
Dextromethorphan (DM)		272.2	Unit	171.1	Unit	5	142	40	X
		272.2	Unit	215.1	Unit	5	142	20	
Verapamil (V)		455.3	Unit	165.1	Unit	5	176	26	X
		455.3	Unit	303.2	Unit	5	176	24	
Methoxyverapamil (MV)		485.3	Unit	165.1	Unit	5	170	28	X
		485.3	Unit	184.2	Unit	5	170	16	
Imipramine (I)		281.2	Unit	86.1	Unit	5	114	12	X
		281.2	Unit	58.1	Unit	5	114	44	
Protriptyline (P)		264.2	Unit	191.1	Unit	5	128	24	X
		264.2	Unit	155.1	Unit	5	128	16	
Amitriptyline (A)		278.2	Unit	91.0	Unit	5	118	24	X
		278.2	Unit	105	Unit	5	118	20	
Trimipramine (T)	X	295.2	Unit	100.1	Unit	5	126	12	X

Table 1: MRM settings determined automatically using MassHunter Optimizer.

Results and Discussion

High-throughput with cassette samples

High-throughput in separations of multiple compounds often requires a compromise between analysis speed and quality of separation – i.e. chromatographic resolution (R_s). **Fig. 2 (A)** illustrates how resolution evolves with flow rate between adjacent peaks in the current cassette. Here, flow of the mobile phase was increased progressively from run-to-run while gradient time was kept constant. (It was shown that greater flow at given gradient time can improve resolution[11]). The range tested was 0.6 to 1.75 mL/min, which resulted in maximum column back pressures (p_{max}) of 470 to 1,160 bar, respectively; backpressure versus flow is also shown in Fig. 2 (A).

Greatest flow yields tremendous speed as depicted in **Fig. 2 (B)**, showing that 1.75 mL/min gave a retention time of the last compound eluting at merely 27 sec (trimipramine = peak 7). Even for a "single compound" analysis, this would be considered extremely high-throughput. In the cassette, however, such ultra high speed may occur at the expense of resolution, as is illustrated here by the critical peak pair methoxyverapamil and imipramine (pair 3 / 4 in Fig. 2). Note: Reported cassette dosing studies often neglect the need for chromatographic separation to avoid ionization suppression effects.

Nevertheless, Fig. 2 (A) shows that advanced technology allows for high speed and high quality separations. No marked changes were observed for resolution at flow rates between 0.6 and 1 mL/min for most adjacent bands, and even the critical peak pair (3 / 4) gave a R_s ~ 1 at 1 mL/min flow. The overlaid MRM-chromatograms in **Fig. 3** show the cocktail of seven being separated within a 1 min chromatographic window (flow 1 mL/min; p_{max} = 740 bar; typically R_s >1; RT_{max} = 42 sec). The corresponding run time was 3 min, which is comparable to run times used in PK analysis for "single compound" studies typically ranging from 3 to 5 min. Cassette dosing analyses often run longer (≥ 6 min). [2-9]

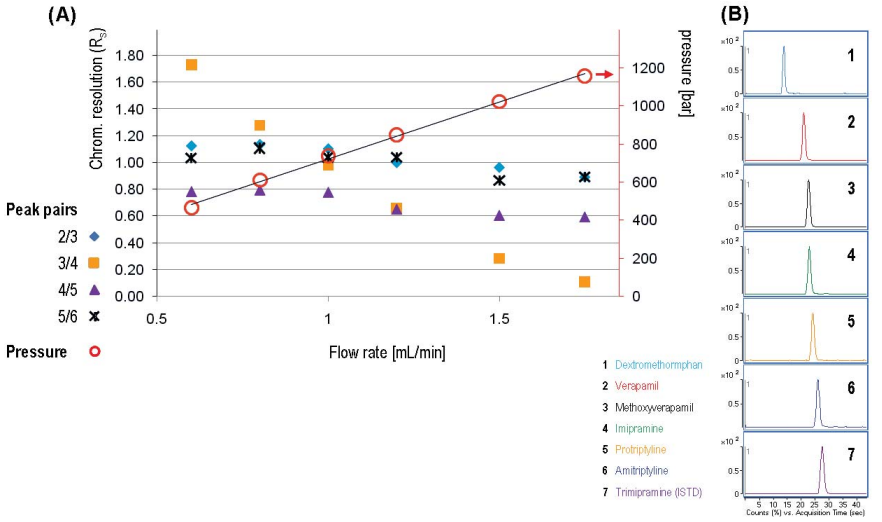

Fig. 2: (A) Plot of chromatographic resolution (R_s/left axis) and maximum column backpressure (right, red axis) versus flow rate. R_s = 1.18 x [RT (2) – RT (1)] / [$w_{0.5}$ (1) + $w_{0.5}$ (2)], where RT = retention time, $w_{0.5}$ = peak width at half height, (1) and (2) corresponds to first and second eluting adjacent peak. (B) MRM-chromatogram showing separation of the seven compounds at 1.75 mL/min flow rate.

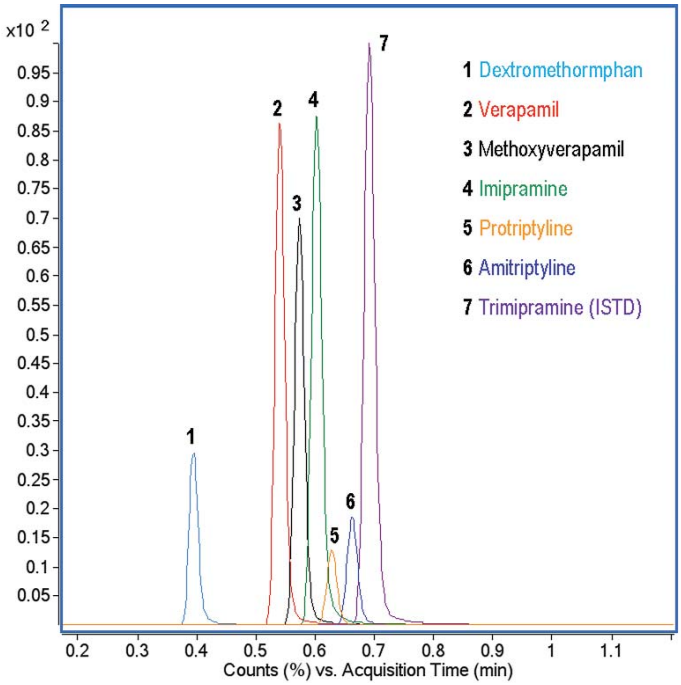

Fig. 3: Representative MRM-chromatogram showing the quality of separation of six compounds plus the internal standard (ISTD). The chromatogram was obtained at mobile phase flow rate of 1 mL/min from a DBS extract. Concentration of drugs in the cassette prior to DBS extraction was 500 ng/mL.

Linearity and LLOQ

Fig. 4 shows calibration plots obtained for imipramine and amitriptyline ranging from lowest limit of quantitation (LLOQ) to 500 ng/mL of blood. The calculation of LLOQ was based on FDA guidelines[12]: the response at LLOQ was at least five times the response in the blank and the response was identifiable, discrete, and reproducible with precision (% RSD) ≤ 20% and accuracy (% bias) 80 to 120%. **Table 2** summarizes LLOQ together with precision and accuracy values for all compounds in the current cocktail, and correlation factors (R^2) of the corresponding calibration curves.

There is excellent linearity since all R^2-values approached unity. LLOQ values determined for the compounds in the cassette ranged from 0.05 - 0.50 ng/mL with good precision and accuracy values. For example, LLOQ (imipramine) = 0.05 ng/mL, precision = 4.3% RSD, accuracy = 107.6%; LLOQ (amitriptyline) = 0.50 ng/mL, precision = 9.5% RSD, accuracy = 99.7%. **Fig. 5 (A)** shows quantifier MRM-traces for both compounds obtained at the LLOQ level (blue) superimposed with the same MRM-traces obtained from the blank extract (purple). There was no marked interfering signal found in the blank for any of the compounds studied at the respective retention time. **Fig. 5 (B)** shows MRM chromatograms of five consecutive injections of LLOQ levels overlaid, which illustrate the typical retention time precision obtained (for amitriptyline and imipramine, 0.066 and 0.088 %RSD, respectively).

Note: Conventional plasma assays usually obtain more than 500 µL of blood per data point. Herein we simulate a study that uses significantly less than 100 µL.[9] Despite this challenge and despite the high-throughput approach, we present LLOQ's that correspond to the lower range reported for traditional PK assays.

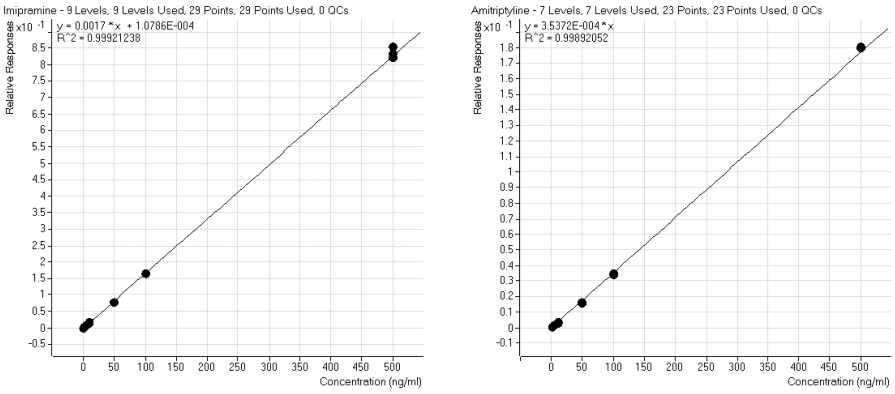

Fig. 4: Representative calibration plots of relative responses (= Response X / response of ISTD) versus nominal concentration in blood (prior to DBS extraction) obtained for imipramine and amitriptyline. The concentration ranges were 0.05 – 500 and 0.5 – 500 ng/mL blood, respectively. Measurements were made n = 5 times for the lowest limit of quantitation and n = 3 times for all other concentrations. Curves were weighted 1/x.

Compound	LLOQ [ng/mL]	Precision N ≥ 5 RSD [%]	Mean Accuracy [%]	R^2
Dextromethorphan	0.50	7.6	101.8	0.998
Verapamil	0.10	8.3	106.4	0.999
Methoxyverapamil	0.10	10.5	93.8	0.999
Imipramine	0.05	4.3	107.6	0.999
Protriptyline	0.50	9.7	97.9	0.997
Amitriptyline	0.50	9.5	99.7	0.999

Table 2: Summary of LLOQ, precision (%RSD), accuracy (%bias) and correlation coefficients (R^2) for the cassette analyzed.

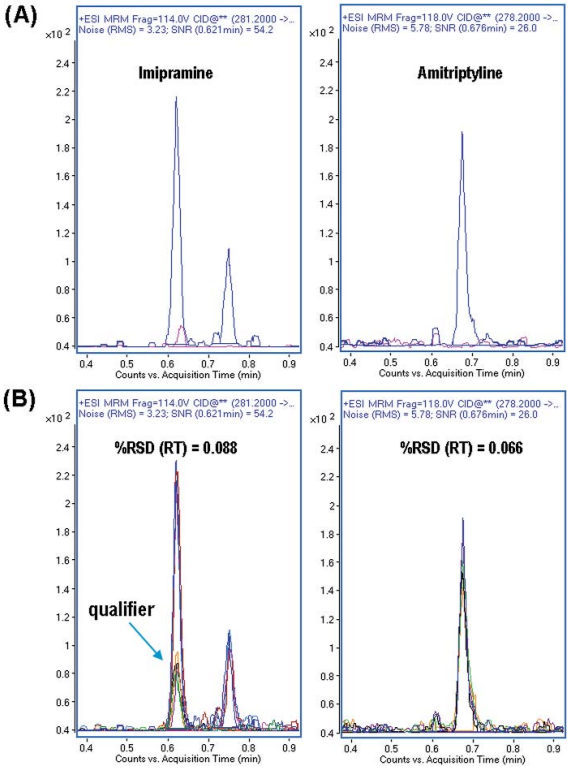

Fig. 5: (A) For imipramine and amitriptyline, representative quantifier chromatogram (blue) obtained for 0.05 and 0.5 ng/mL blood (prior to DBS extraction), respectively. Overlaid quantifier chromatograms obtained from the blank extract (purple). (B) MRM-chromatograms of the five consecutive injections at LLOQ level overlaid. For imipramine, quantifier and qualifier traces are shown. Values given are retention time (RT) precision (%RSD).

Intra-day precision of DBS extractions

Intra-day precision and accuracy of the current method were tested by extraction and analysis of three replicates at three concentrations (0.5, 10, 100 ng/mL blood); full validation was beyond the scope here. **Fig. 6** shows representative plots of relative response versus concentration (log-log scale) obtained for imipramine and amitriptyline. The replicate data points are shown as superimposed blue triangles. **Table 3** summarizes the results for all compounds.

Values for precision and accuracy generally were well within the 15% limits required by recognized acceptance criteria.[12] Precision and accuracy found here at the 0.50 ng/mL level further confirm some of the LLOQ values given in Table 2.

Matrix effect

To assess ion suppression due to matrix components associated with DBS, responses of ISTD in post-extraction spiked DBS-extracts were compared with those obtained for ISTD of the same concentration in reference solution. The matrix factor found was 0.7.

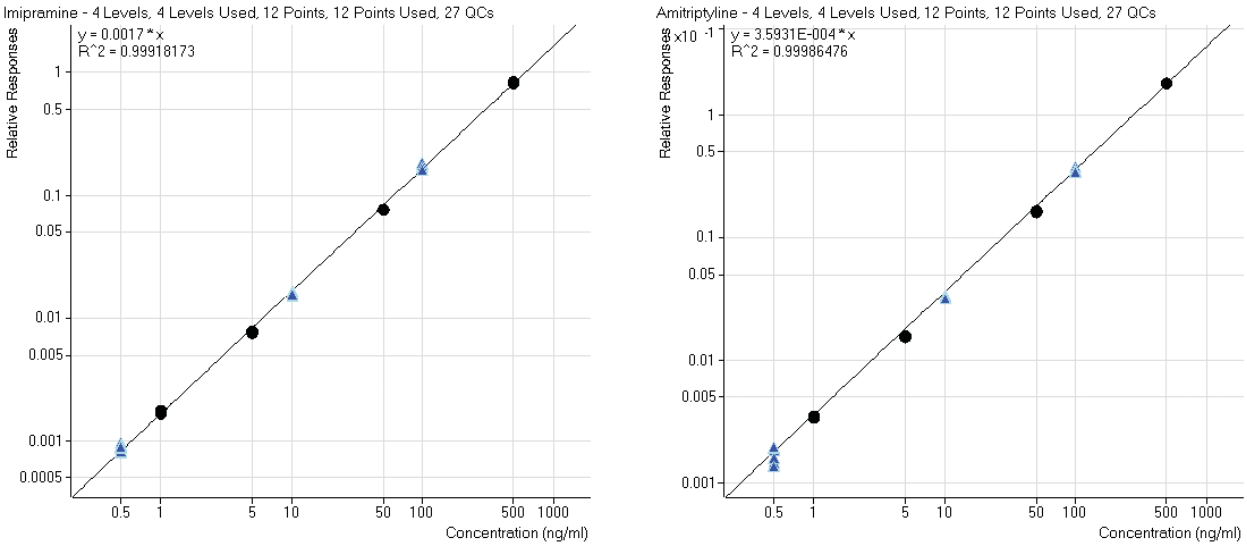

Fig. 6: Plots depicting intra-day precision of the method. Calibration plots of relative responses versus concentration (log-log scale) in blood prior to DBS extraction for imipramine and amitriptyline are superimposed with the results obtained from replicate DBS extractions at three concentrations (0.5, 10, 100 ng/mL blood) (blue triangles).

Compound	Nominal Conc. [ng/mL]	Av. measured Conc. [ng/mL]	Intra-day P. RSD [%]	Av. Accuracy [%]
Dextromethorphan	0.50	0.48	13.8	96.6
	10.00	10.38	2.0	107.6
	100.00	111.71	2.3	111.4
Verapamil	0.50	0.54	7.5	108.6
	10.00	11.04	1.6	109.5
	100.00	111.61	2.9	111.6
Methoxyverapamil	0.50	0.50	10.0	100.1
	10.00	10.40	2.0	104.0
	100.00	109.12	3.2	109.1
Imipramine	0.50	0.46	6.4	92.0
	10.00	9.43	2.3	94.3
	100.00	104.72	4.5	104.7
Protriptyline	0.50	0.47	12.9	94.5
	10.00	8.79	3.0	87.9
	100.00	98.18	5.9	98.2
Amitriptyline	0.50	0.47	11.6	95.7
	10.00	9.34	1.9	93.4
	100.00	102.16	4.8	102.2

Table 3: Summary of results from intra-day precision (%RSD) and accuracy (%bias) obtained from replicate extractions of the cassette at three concentrations. Precision was determined by calculating %RSD for the replicates within the same concentration and accuracy by calculating %deviation from the theoretical (nominal) concentration.

Conclusions

We demonstrated that advanced instrumentation is the key to ultra-high productivity analysis without having to compromise quality of the chromatographic separation or sensitivity.

- The 1290 Infinity LC has a small gradient delay volume and can be operated at ultra high pressures and flow rates.
- The 6460 Triple Quad is equipped with the Agilent Jet Stream technology, which allows for high flow rates without the need to split.
- The ZORBAX RRHD sub-2-µm particle column provides chromatographic quality even under fast separation conditions.
- We were able to use flow rates of up to 1.75 mL/min approaching pressures of 1,200 bar.
- We analyzed DBS extracts that contained seven drugs in less than 50 sec and still maintained excellent separation quality.
- We showed linearity and high-end LC/MS sensitivity under these ultra high-throughput conditions.

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