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QUANTITATIVE DETERMINATION OF TULOBUTEROL AND ITS METABOLITES IN HUMAN URINE BY MASS FRAGMENTOGRAPHY

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SUMMARY

A method is described for the simple and simultaneous determination of tulobuterol and its metabolites in human urine by gas chromatography—mass spectrometry. Quantification was achieved by single-ion monitoring at *m/e* 86 derived from trimethylsilyl-tulobuterol and its metabolites using a column packed with a mixed phase, 2% OV-1—2% QF-1 (1 : 1, w/w). The detection limits were estimated to be 2 ng/ml in urine for tulobuterol and 5 ng/ml for metabolites, respectively.

INTRODUCTION

Tulobuterol (Fig. 1) is one of the sympathomimetic amines synthesized by Koshinaka et al. [1], which has intensive bronchodilatory activity.

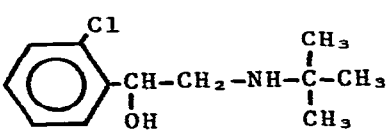

Fig. 1. Structure of tulobuterol.

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Determination of these sympathomimetic amines in biological fluids has mostly been undertaken using radio-labelled compounds [2—4], because of the relatively low concentration of these drugs in the body. Recently, quantitative analysis of salbutamol and terbutaline in plasma using gas chromatography (GC)—mass fragmentography has been reported by Martin and co-workers [5, 6] and Leferink et al. [7].

In the present work, we have described the simple and simultaneous determination of tulobuterol and its possible metabolites, predicted from metabolism in the rat [8], in human urine after the therapeutic dosage of tulobuterol.

EXPERIMENTAL

Materials

All reference samples of tulobuterol·HCl (I), its metabolites [4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV), 5-hydroxytulobuterol (V)], and 4-methoxytulobuterol (internal standard, IS) were prepared in this laboratory according to the published method [1]. N,O-Bis(trimethylsilyl)acetamide (BSA) was obtained from Nakarai Chemical (Kyoto, Japan) and glusulase from Endo Labs. (Garden City, NY, U.S.A.). Other reagents and solvents were of analytical grade and were used without further purification.

Gas chromatography

A Hitachi Model 063 gas chromatograph with a flame ionization detector was used. The glass columns were packed with 1.5% SE-30 (Chromosorb G AW DMCS, 60—80 mesh), 5% SE-52 (Chromosorb W AW DMCS, 60—80 mesh), 2% OV-1 (Chromosorb W AW DMCS, 100—120 mesh), 1.5% OV-17 (Chromosorb G AW DMCS, 60—80 mesh), 2% OV-225 (Gas-Chrom Q, 80—100 mesh), 2% F-50 (Chromosorb W AW DMCS, 80—100 mesh), 2% XF-1150 (Chromosorb W AW DMCS, 80—100 mesh), 2% Dexil 300GC (Chromosorb W AW DMCS, 80—100 mesh), 2% QF-1 (Gas-Chrom Q, 80—100 mesh), mixed phase of 2% OV-1 (Chromosorb W AW DMCS, 100—120 mesh) and 2% QF-1 (Gas-Chrom Q, 80—100 mesh) (1 : 1, w/w).

The flow-rates for the carrier gas (nitrogen), hydrogen, and air were 40, 30, and 580 ml/min, respectively. The temperature for both the injector and detector was 190°C.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry (GC—MS) was carried out on a system of JMS D-300 consisting of a JGC-20KD gas chromatograph and JMA-2000 data system (JEOL, Tokyo, Japan).

GC separation was carried out by using a glass column (3 m × 2 mm I.D.) packed with mixed phase, 2% OV-1—2% QF-1 (1 : 1, w/w). Helium was used as the carrier gas (flow-rate 33 ml/min) and ammonia at a pressure of ca. 1.0 Torr was used as the chemical ionization (CI) reagent gas. The temperature of the column oven was 165°C for the trimethylsilylation and 190°C for the O-TMS, N-TFA derivatization. In addition, the temperatures of the injection port, separator, and ion source were 200°C, 290°C and 150°C, respectively. The ionization energy was 70 eV for the electron impact (EI) mode and 210 eV for the CI mode, and the trap current was 300 μA for both modes.

Drug administration and sample collection

Three healthy male volunteers received an oral dose of 1 mg tulobuterol·HCl (as a tablet) and the urine samples were collected separately for 0—4, 4—8, 8—12, 12—24 and 24—32 h after dosing and stored at −20°C until analysed.

Extraction and derivatization

Conjugated metabolites. To 4 ml of urine in a 15-ml glass-stoppered tube which contained 2.5 μg of 4-methoxytulobuterol as internal standard, was added 0.75 ml of 1 *M* acetate buffer and the pH was adjusted to 5.1—5.3. After the addition of 0.25 ml of enzymatic solution (glusulase), the sample was incubated overnight at 37°C. After cooling, 2 g of NaCl and 1 ml of NH_4Cl—NH_4-OH buffer (1.0 *M*, pH 10.0) were added and the pH was adjusted with 1 *N* NaOH to 9.5. The sample was shaken with ethyl acetate—acetone (3:1, v/v) for 10 min and centrifuged for 5 min at 2500 *g*; 3 ml of the organic layer was transferred to another tube and evaporated to dryness in a water-bath (40—50°C) under reduced pressure. The residue was dissolved in 250 μl of ethyl acetate, and 250 μl of BSA was then added. The contents of the tube were allowed to react at room temperature for at least 1 h. This final solution was injected into GC—MS system.

Unconjugated metabolites. Extraction and derivatization of unconjugated metabolites were carried out in the same way, omitting the glusulase incubation.

RESULTS AND DISCUSSION

In order to evaluate the GC properties and separation of compounds I—V, an aliquot of reference compounds was derivatized and analysed using various columns (Table I). As a result, symmetrical peaks and good separation could be obtained for the TMS derivatives of I—V when a glass column packed with mixed phase, 2% OV-1—2% QF-1, was used (Fig. 2.).

Fig. 3 shows the CI and EI spectra of the TMS derivatives (I—V). The quasi molecular ion $(M+1)^+$ and the fragment ion $(M-OTMS)^+$ were present in the CI spectra, while in the EI spectra of these compounds a base peak appeared at

TABLE I

RETENTION TIMES OF TMS DERIVATIVES

II= 4-Hydroxytulobuterol; III= 3-hydroxytulobuterol; V= 5-hydroxytulobuterol.

Column system				Retention time (min)		
Packing support	Mesh	length (m) × mm I.D.	Temperature (°C)	II	III	V
1.5% SE-30 Chromosorb G AW DMCS	60—80	2 × 3	165	12.0	12.0	10.7
5% SE-52 Chromosorb W AW DMCS	60—80	2 × 3	190	15.8	15.8	14.3
2% OV-1 Chromosorb W AW DMCS	100—120	2 × 3	170	18.1	17.3	15.7
1.5% OV-17 Chromosorb G AW DMCS	60—80	2 × 3	165	12.3	12.3	11.3
2% OV-225 Gas-Chrom Q	80—100	3 × 3	170	6.7	6.7	6.0
2% F-50 Chromosorb W AW DMCS	80—100	3 × 3	180	16.8	15.8	14.3
2% XF-1150 Chromosorb W AW DMCS	80—100	0.5 × 3	150	4.9	4.9	4.3
2% Dexil 300GC Chromosorb W AW DMCS	80—100	1 × 3	160	7.6	7.4	6.3
2% QF-1 Gas-Chrom Q	80—100	2 × 3	130	14.3	12.8	12.3
2% OV-1 − 2% QF-1 (1:1, w/w)		3 × 3	140	22.0	20.1	18.7

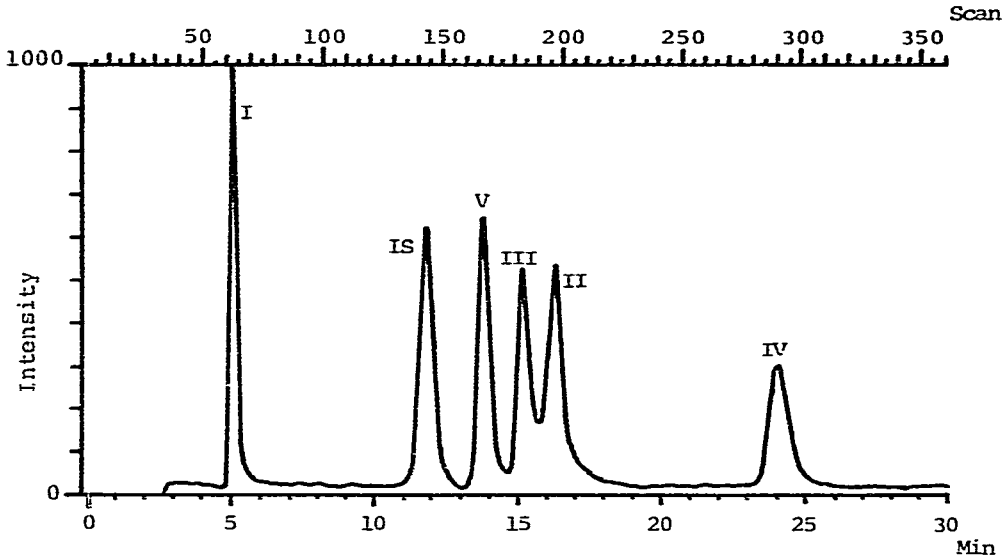

Fig. 2. Total ion current chromatogram of tulobuterol (I), 4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV), 5-hydroxytulobuterol (V) and 4-methoxytulobuterol (internal standard, IS) as their TMS derivatives.

m/e 86 which had resulted from fragmentation by β-cleavage. We chose the single-ion monitoring method at *m/e* 86 for the quantitative analysis of compounds I—V, because we could not achieve reproducible ionization in the CI measurement.

4-Methoxytulobuterol was evaluated for use as the internal standard, because the TMS derivative of this compound gave mass spectra very similar to that of the corresponding compounds I—V and the GC retention time was different from those of I—V.

We also examined other derivatizing methods such as acylation by trifluoroacetic anhydride (TFA) or pentafluoropropionic anhydride [9] and a two-step derivatization by bis(trimethylsilyl)trifluoroacetamide and TFA [10], but these methods were unstable and/or time-consuming compared to TMS derivatization by BSA.

Calibration graphs of compounds I—V in human urine are shown in Fig. 4. The plots of concentration vs. peak area ratio relative to internal standard added to the sample were found to be linear over the range 5—300 ng/ml urine.

The analytical recoveries and standard deviations for compounds I—V were reasonable, as summarized in Table II. This also indicates that the method is very precise and accurate. Futhermore there was no influence due to non-specific interference at *m/e* 86, which is a relatively low mass, from endogenous material of urine extracts.

Fig. 5 illustrates single-ion monitoring from urine after an oral dose of a tulobuterol tablet (1 mg). The major peaks of I, II, V, and two unidentified metabolites were detected in urine before enzymatic hydrolysis, although a trace peak of III less than 5 ng/ml appeared in the chromatogram. On the other hand, from the urine sample after treatment with glusulase, metabolites III and IV were apparently detected in addition to I, II and V.

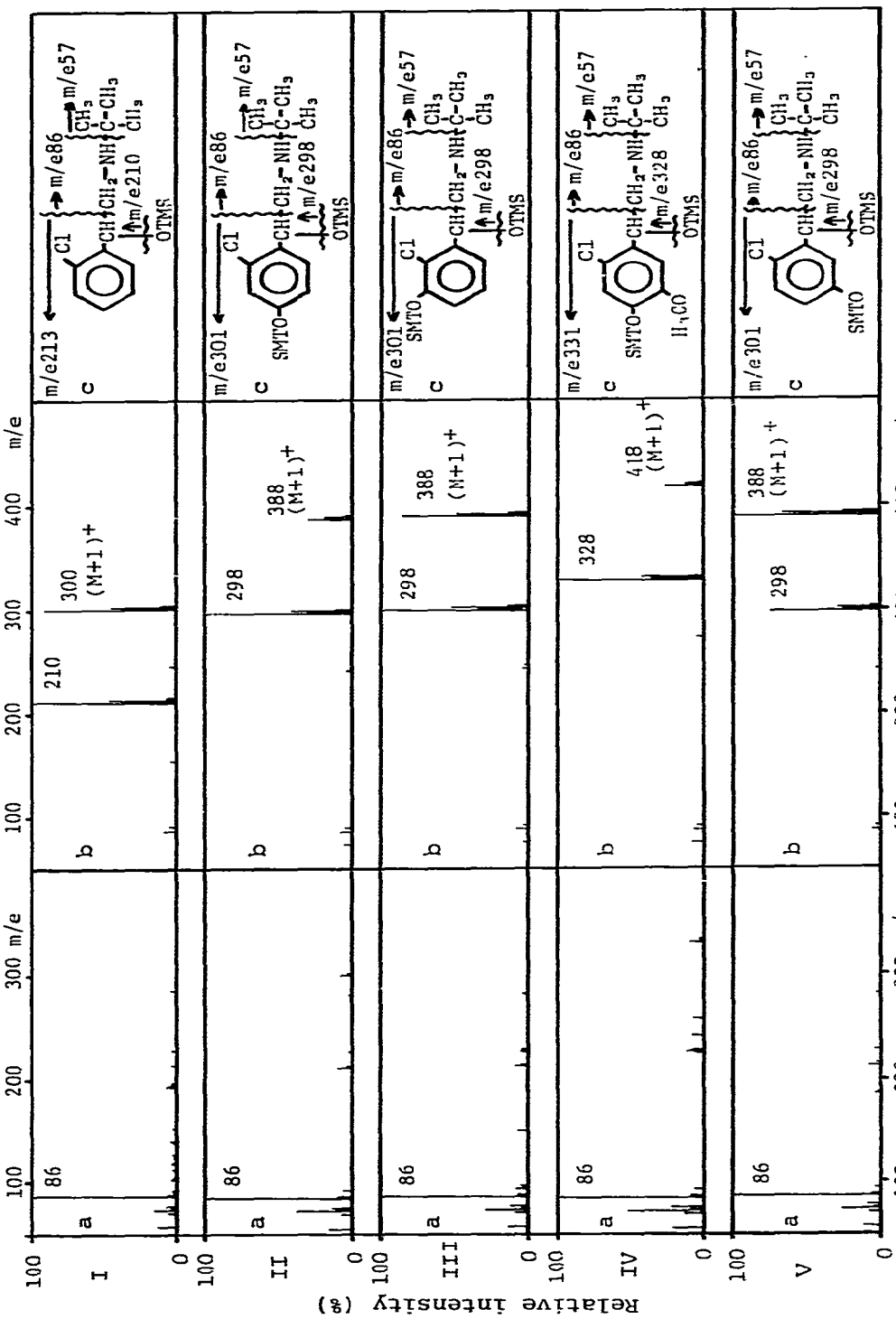

Fig. 3. Mass spectra and fragmentation of tulobuterol (I), 4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV) and 5-hydroxytulobuterol (V) as their TMS derivatives. (a) EI mass spectra; (b) CI mass spectra; (c) fragmentation.

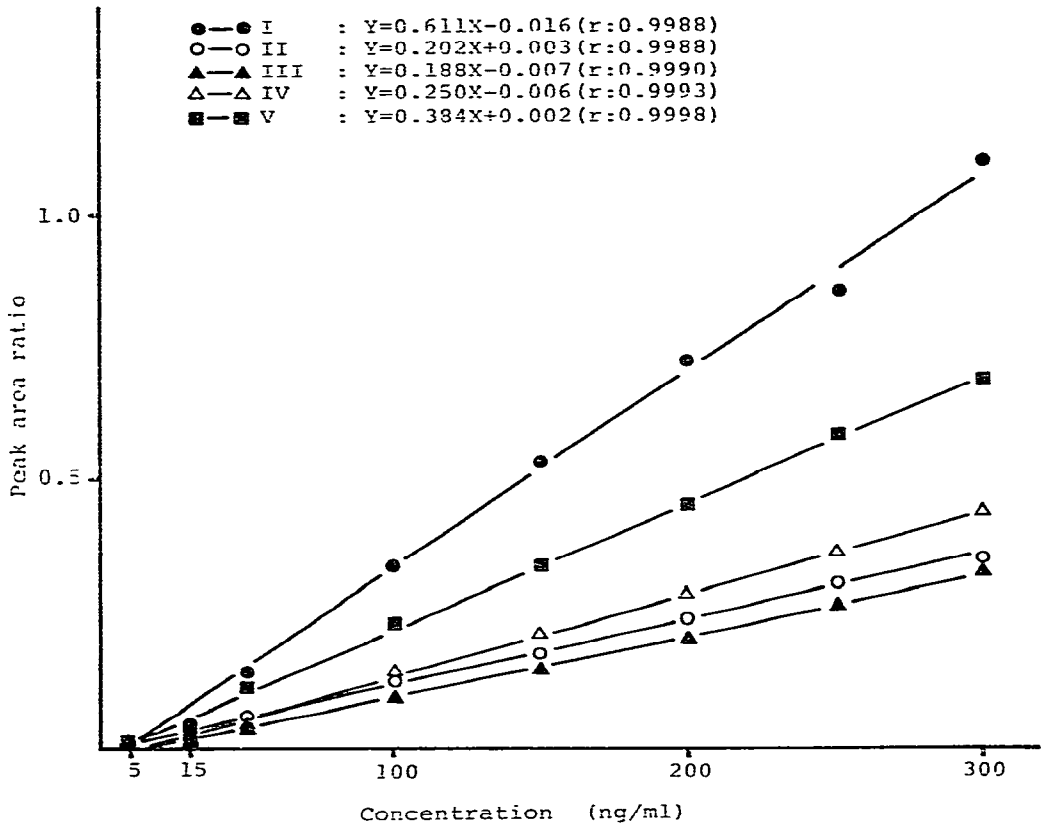

Fig. 4. Calibration curves for tulobuterol (I), 4-hydroxy- (II), 3-hydroxy- (III), 4-hydroxy-5-methoxy- (IV) and 5-hydroxytulobuterol (V) in human urine.

TABLE II

RECOVERIES OF TULOBUTEROL (I), 4-HYDROXY- (II), 3-HYDROXY- (III), 4-HYDROXY-5-METHOXY- (IV) AND 5-HYDROXYTULOBUTEROL (V) IN HUMAN URINE

$n = 6$.

Metabolite	Added (ng/ml)	Found (ng/ml)	Recovery (% ± S.D.)
I	100	95	94.7±6.64
	250	273	109.2±6.76
II	100	95	95.0±3.52
	250	230	92.1±3.90
III	100	96	95.7±5.31
	250	255	101.9±3.30
IV	100	94	93.5±7.15
	250	246	98.2±6.30
V	100	100	100.0±3.24
	250	249	99.7±2.57

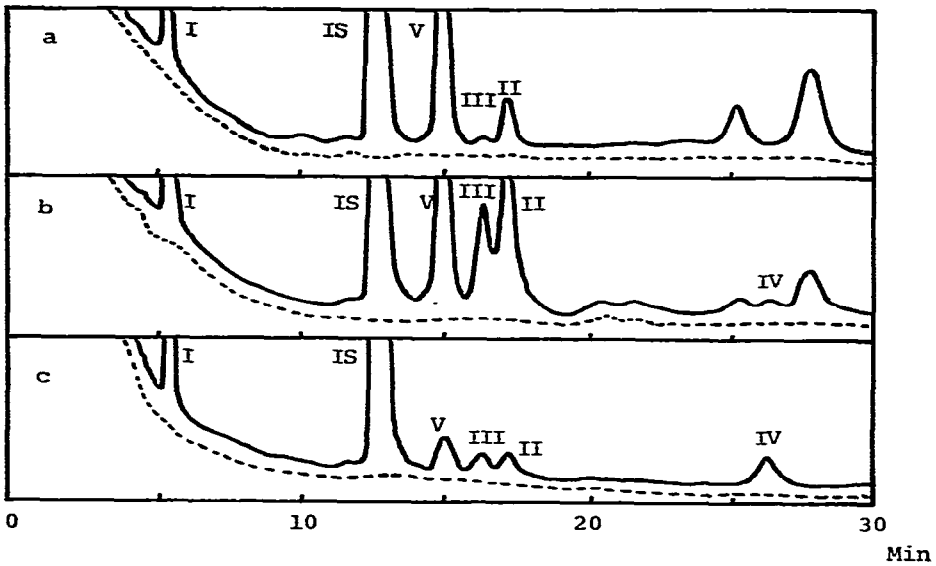

Fig. 5. Single-ion (*m/e* 86) monitoring of urine extracts. (a) Untreated urine (subject O.K., 0—4 h after dose); (b) treated urine (subject O.K., 0—4 h after dose) with glusulase; (c) blank urine spiked with compounds I—V (50 ng/ml urine) and 4-methoxytulobuterol [internal standard (IS), 500 ng/ml urine]. Dotted lines, tracings for blank urine.

TABLE III

URINARY EXCRETION OF TULOBUTEROL AND ITS METABOLITES AFTER ORAL ADMINISTRATION OF 1 mg OF TULOBUTEROL TO HUMAN SUBJECTS

I = Tulobuterol; II = 4-hydroxytulobuterol; III = 3-hydroxytulobuterol; IV = 4-hydroxy-5-methoxytulobuterol; V= 5-hydroxytulobuterol. N.D.= Not detected.

Subject	Time(h)	Dose in urine (%)									
		I		II		III		IV		V	
		Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
M.O.	0—4	7.4	18.0	N.D.	0.2	N.D.	0.1	N.D.	0.1	0.2	0.2
	4—8	5.5	11.1	N.D.	0.2	N.D.	0.0	N.D.	0.1	0.2	0.1
	8—24	9.6	20.2	N.D.	0.7	N.D.	0.4	N.D.	0.3	0.3	1.0
	24—32	1.0	1.2	N.D.	0.2	N.D.	N.D.	N.D.	0.1	0.2	0.1
	Total	23.5	50.5	–	1.3	–	0.5	–	0.6	0.9	1.4
I.T.	0—4	5.0	8.1	1.2	1.9	N.D.	3.4	N.D.	N.D.	0.8	1.0
	4—8	2.6	4.2	0.6	1.7	N.D.	2.4	N.D.	N.D.	0.9	1.9
	8—24	3.8	3.0	1.1	0.8	N.D.	2.0	N.D.	0.4	1.2	1.2
	24—32	0.6	N.D.	N.D.	0.2	N.D.	1.1	N.D.	N.D.	0.2	0.5
	Total	12.0	15.3	2.9	4.6	–	8.9	–	0.4	3.1	4.5
O.K.	0—4	2.5	5.7	1.0	2.8	N.D.	2.8	N.D.	0.2	1.2	2.3
	4—8	1.7	3.0	0.5	1.9	N.D.	2.0	N.D.	0.3	1.1	2.8
	8—24	2.0	2.9	0.9	1.7	N.D.	2.0	N.D.	0.6	1.4	4.0
	24—32	0.8	N.D.	N.D.	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	0.3
	Total	7.0	11.6	2.4	6.6	–	6.8	–	1.1	3.7	9.4

The overall analytical results are given in Table III, and indicate that the main metabolic pathway of tulobuterol in man is the ring-hydroxylation, but a considerable amount of unchanged drug is excreted in urine.

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