J. Pharm. Pharmacol. 1988, 40: 552–557
Received March 4, 1988

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Strategy to Assess the Role of (Inter)active Metabolites in Pharmacodynamic Studies In-vivo: a Model Study with Heptabarbital

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Abstract—The purpose of this investigation was to develop a universal experimental strategy by which the role of (inter)active metabolites in in-vivo pharmacodynamic studies can be examined. Heptabarbital was chosen as a model drug and several pharmacokinetic variables which may affect in-vivo concentration-pharmacological response relationships were examined. Adult female rats received an i.v. infusion of the drug at one of three different rates (0·225–1·50 mg min^{-1}) until the animals lost their righting reflex (after 11 ± 1 to 88 ± 8 min of infusion). The serum concentration of the drug at onset of loss of righting reflex (LRR) increased slightly with increasing infusion rate. The drug concentrations in brain tissue and cerebrospinal fluid (CSF), (mean ± s.d.: 67 ± 5 mg kg^{-1} and 24 ± 4 mg L^{-1}, respectively, for the lowest infusion rate) were not affected by the infusion rate. The possible contribution of (inter)active metabolites to the pharmacological response of heptabarbital was determined by administration of different i.v. bolus doses (14·1–22·5 mg) resulting in widely differing sleeping-times (7 ± 3 to 119 ± 20 min). The concentrations of heptabarbital in serum, brain tissue and CSF at offset of LRR (mean ± s.d.: 77 ± 8 mg L^{-1}, 76 ± 7 mg kg^{-1} and 29 ± 5 mg L^{-1}, respectively, for the highest dose) were not affected by the administered dose. Kinetic analysis of the relationship between dose and the duration of the pharmacological response revealed an elimination half-life of heptabarbital of $2·8 \pm 0·2$ h, which is in close agreement with the value determined on the basis of the plasma concentration vs time profile following administration of 22·5 mg i.v. ($2·8 \pm 0·4$ h). In a separate investigation no statistically significant differences were observed in heptabarbital concentrations at onset of LRR during an i.v. infusion (0·563 mg min^{-1}) and at offset of LRR following an i.v. bolus dose (22·5 mg; sleeping time: 100 ± 20 min). These results show that (a) there is a rapid equilibration between the concentrations of heptabarbital (heptabarbitone) in CSF and those at the site of action (i.e. the CSF compartment is pharmacokinetically indistinguishable from the site of action), (b) metabolites do not interfere with the pharmacological effect of heptabarbital and (c) within the time-frame of the experiments there is no development of 'acute' tolerance to the anaesthetic effect of heptabarbital. It is concluded that a combination of determination of the concentrations at offset of a certain pharmacological effect following administration of different drug doses, and evaluation of the dose vs duration of pharmacological response relationship, can be a powerful tool in examining the role of unknown (inter)active metabolites.

It is well-known that considerable differences exist in the 'sensitivity' to the effects of drugs between individual subjects. A great amount of effort has been devoted to studies trying to explain these differences, e.g. due to an altered physiological status, pharmacokinetically. These studies, however, generally leave potential effects of diseases on pharmacodynamics out of consideration (Levy 1983, 1984). Especially in the field of drugs with effects on the central nervous system, studies which unequivocally report on relationships between drug concentration in plasma or in the biophase and the intensity of the pharmacological response are scarce. Most likely this is due to the many potentially complicating pharmacokinetic factors that are associated with in-vivo pharmacodynamic studies. These include: (a) differences in distribution, i.e. between the blood plasma, in which drug concentrations are usually determined, and the site of action, (b) accumulation of active and/or interactive metabolites, (c) differences in the disposition and pharmacological activity of the enantiomers of drugs which are racemic mixtures and (d) the development of acute tolerance (Dingemanse et al 1988). Of these complicating pharmacokinetic factors the role of (inter)active metabolites presents a special problem since the complete metabolite profile of most drugs is unknown. In addition, known metabolites are rarely available in sufficient quantities for direct pharmacological testing.

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The purpose of the present investigation was to examine a universal experimental strategy by which the role of unknown metabolites in in-vivo pharmacodynamic studies can be determined indirectly, using heptabarbital as a model drug. In this type of study several different potentially confounding pharmacokinetic factors have to be taken into consideration. The experiments described include, therefore, determination of the site of action of heptabarbital by examining the concentrations at onset of loss of righting reflex (LRR) during intravenous infusions at different rates, determination of the role of (inter)active metabolites by comparison of the concentrations at offset of LRR following administration of different doses, and determination of the development of *acute* tolerance by comparison of the concentrations of heptabarbital in cerebrospinal fluid at onset and offset of loss of righting reflex.

Materials and Methods

Chemicals

Heptabarbital (5-ethyl-5-(1′-cycloheptenyl)barbituric acid)

was a generous gift from Ciba-Geigy (Arnhem, The Netherlands). Hexobarbital (1,5-dimethyl-5-(1′-cyclohexenyl)barbituric acid) and secobarbital (5-allyl-5-(1-methylbutyl hexenyl methyl butyl-barbituric acid) were purchased from Brocacef (Maarssen, The Netherlands).

Dichloromethane, light petroleum (bp 40–60°C) and ethyl acetate were obtained from J. T. Baker (Deventer, The Netherlands) and were freshly redistilled. Acetonitrile (p.a. grade) was obtained from Merck (Darmstadt, FRG).

Animals

Female SPF Wistar rats (160–210 g) from a laboratory breed and maintained on a commercially available diet (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) were used. The rats had indwelling cannulas implanted in the right jugular vein (Weeks & Davis 1964) under light ether anaesthesia one day before the experiment. From the night before the study the animals received no food, but they had free access to water. After the operation they were housed individually in plastic cages. To minimize the influence of a possible diurnal rhythm in brain sensitivity and/or metabolism rate (Roberts et al 1970), all experiments were carried out between 1000 and 1400 h, with the exception of the determination of the pharmacokinetics which took a longer time.

Pharmacokinetics

The pharmacokinetics of heptabarbital were determined in five rats after i.v. injection of the drug (22·5 mg/rat) and serial collection of nine intravenous 0·2 mL mainstream blood samples over 8 h. The injection solution (which also was used for the other experiments) consisted of heptabarbital dissolved in an equimolar quantity of 0·1 M NaOH and made up with distilled water to a concentration of 15 mg in 1 mL. The solution was freshly prepared daily. Serum was separated and stored at −20°C until analysed.

Infusion rate experiment

The effect of the infusion rate on heptabarbital concentrations in different body compartments at onset of loss of righting reflex (LRR) was determined by i.v. infusion (Unita I infusion pump, Braun, Melsungen, FRG) of drug at three different rates: 0·225, 0·563 and 1·50 mg min^{-1}. The experiment was performed over two successive days with the animals being divided randomly into the different groups. Each infusion rate group consisted of 5 to 8 animals. During drug infusion body temperature was maintained at 37°C by placing the animals on an isothermal heating-plate. The onset of drug effect was determined as described previously (Danhof & Levy 1984), at which time CSF, blood (for serum) and brain tissue were obtained for determination of heptabarbital concentration by gas chromatography or high-pressure liquid chromatography.

Sleeping-time experiment

To three groups of 9 rats each, heptabarbital was administered as a short infusion (5·63 mg min^{-1}) in a dose of 14·1, 17·8 and 22·5 mg, respectively. This experiment was conducted over three successive days with the three different doses tested on each day. At offset of LRR, samples of body fluids and tissue were obtained in the aforementioned way.

Onset versus offset experiment

Sixteen animals were randomly allocated to two groups, one of which received a heptabarbital infusion at 0·563 mg min^{-1} and subsequently was treated as in the infusions experiment. The animals in the other group received a dose of 22·5 mg heptabarbital in a 4 min infusion. At offset of LRR the usual samples were collected for determination of the heptabarbital concentration.

Analytical procedures

Serum protein binding was determined by equilibrium dialysis (Dianorm rotator, Diachema, Abcoude, The Netherlands) at 37°C against an equal volume of 0·13 M phosphate buffer, pH 7·4, containing heptabarbital, 40 mg L^{-1} and with a semipermeable membrane having a molecular weight cutoff of 10 000. Dialysis was performed during 2 h, by which time equilibrium had been shown to be established. In a preliminary experiment the extent of serum protein binding of heptabarbital was shown to be independent of drug concentration in the range 20 to 200 mg L^{-1}.

Initially, heptabarbital concentrations were determined by capillary gas chromatography. Hexobarbital was used as the internal standard. A solution of 8 μg of hexobarbital in 100 μL ethanol was added to 50–100 μL of serum, CSF or dialysis solution, followed by the addition of 400 μL water. After extraction for 20 s on a whirlmixer with 5 mL of dichloromethane–light petroleum (1 : 1) and centrifugation (5 min at 3000 rev min^{-1}), the organic layer was evaporated to dryness under a gentle stream of nitrogen. To determine heptabarbital concentrations in brain, one hemisphere was accurately weighed and homogenized in 1·2 mL ethanol, containing 48 μg hexobarbital as the internal standard. 0·50 mL of the homogenate was extracted twice with 5 mL dichloromethane–light petroleum. In all determinations the residue was finally dissolved in 100 μL of ethyl acetate and 0·5–0·8 μL were evaporated on the tip of an injection needle, which was introduced into the gas chromatograph (Hewlett Packard model 5710A) equipped with an electron capture detector and a solid injection system.

A capillary column (length, 15 m; inside diameter 0·35 mm) made of Pyrex glass was used. The support layer was Tullanox (silanized fused silica), particle size 10 μm (Cabot Corp., Boston, MA, USA) and the stationary phase was OV-1701 (0·2% w/v in pentane). The operating temperatures were: injection port, 300°C; column 210°C; and detector 350°C. The flow rate of the carrier gas (helium) was 5 mL min^{-1} and of the auxiliary gas (argon-methane 95:5) was 25 mL min^{-1}.

Peak height ratios of heptabarbital and the internal standard were used to calculate drug concentrations with the aid of calibration graphs. The extraction yield appeared to be constant and linear with concentration, with a mean value of 90% for the different body fluids. The method was reproducible and had satisfactory precision: the standard deviations of single determinations of concentrations in the range of 20–200 mg L^{-1} or mg kg^{-1} never exceeded the value of 7% ($n = 5$).

In an attempt to determine metabolite(s) of heptabarbital, a high-pressure liquid chromatographic method was set up, as described by Danhof & Levy (1985).

The HPLC system (Waters Associates, Milford, MA, USA) consisted of a M-45 pumping device, a WISP 710B automatic sample injector, a Z-module containing a Radial-Pak C-18 cartridge, and a M-440 UV detector at 254 nm. Data processing was performed with a Hewlett-Packard 3390A reporting integrator.

To compare the different chromatographic techniques, some series of serum and brain samples were analyzed by both methods. A strong correlation ($r > 0 \cdot 95$) was found, with a slope close to unity, so the results obtained by these two assays were regarded to be interchangeable.

Data analysis

Serum elimination half-life was determined by linear regression of the log serum concentration versus time curves. Areas under the curve (AUC) were calculated with the aid of the trapezoidal rule method. Total clearance was estimated as Dose/AUC and the apparent volume of distribution as Dose/AUC*k, where k is the elimination rate constant. The results of the pharmacodynamic experiments were assessed statistically by analysis of variance and by the Newman-Keuls test where appropriate.

Elimination half-life and minimum effective dose based on the sleeping-time experiment were calculated from a duration of effect versus log dose plot (Levy 1966).

Results

Pharmacokinetics in serum

All serum concentration vs time curves in individual rats exhibited a log-linear disappearance of heptabarbital and the pharmacokinetic parameters derived are presented in Table 1. When assayed by HPLC an additional peak was present in the chromatogram, which initially increased and subsequently decreased with time. This suggests that it represented a metabolite of heptabarbital.

Table 1. Comparison of pharmacokinetic parameters derived from the pharmacokinetic and the sleeping-time experiment.

	Kinetic	Dynamic
V_{ss} (L kg^{-1})	0·80±0·11	—
CL (L h^{-1} kg^{-1})	0·23±0·05	—
k (h^{-1})	0·25±0·04	0·25±0·02
$t_{\frac{1}{2}}$ (h)	2·8±0·4	2·8±0·2

Results are reported as mean ± s.d.

Table 2. Effect of infusion rate on infusion time, total dose and concentrations of heptabarbital at onset of loss of righting reflex.

	Infusion rate (mg min^{-1})		
	0·225	0·563	1·50
No. of animals	8	7	5
Body weight (g)	179±13	177±15	177±12
Infusion time (min)	88±8	31±5	11±1*
Total dose (mg kg^{-1})	111±13	93±6	91±9**
Serum concn (mg L^{-1})			
Total drug	89±10	103±5	108±11**
Free drug	42±10	51±7	54±6
Brain concentration (mg kg^{-1})	67±5	69±7	71±12
CSF concentration (mg L^{-1})	24±4	26±2	23±4
Protein binding in serum (%)	53±8	51±7	49±4

Results are reported as mean ± s.d.
* Infusion time is significantly influenced by the infusion rate ($P < 0 \cdot 001$).
** Significant differences are only found between the lowest and highest infusion rates ($P < 0 \cdot 05$).

Table 3. Effect of administered dose on sleeping-times and heptabarbital concentrations at offset of loss of righting reflex.

	Dose (mg)		
Parameter	14·1	17·8	22·5
No. of animals	9	9	9
Body weight (g)	173±16	173±11	171±15
Duration of LRR (min)	7±3	66±18	119±20*
Serum concn (mg L^{-1})			
Total drug	81±6	77±9	77±8
Free drug	37±12	33±17	37±12
Brain concentration (mg kg^{-1})	87±7	74±10	76±7
CSF concentration (mg L^{-1})	31±4	32±6	29±5
Peak height ratio metabolite	0·08±0·05	0·53±0·14	0·77±0·12*

Results are reported as mean ± s.d.
* Duration of LRR and peak height ratio of metabolite are significantly influenced by the administered dose ($P < 0 \cdot 001$).

Infusion rate experiment

Intravenous infusion of heptabarbital at the three different rates resulted in a smooth and accurately determinable onset of LRR. The pharmacological endpoint occurred at 11 ± 1, 31 ± 5 and 88 ± 8 min (mean ± s.d.), with decreasing infusion rate (Table 2). The total amount of drug administered ranged from 91 ± 9 to 111 ± 13 mg kg^{-1} and was highest for the slowest infusion rate. With regard to the concentrations of heptabarbital at the onset of LRR, the only significant difference was observed between the two extreme infusion rates with respect to total serum concentration (Table 2). Free serum, brain and CSF concentrations at onset of effect appeared to be independent of the infusion rate. Also serum protein binding was not affected by the infusion rate.

Sleeping-time experiment

The sleeping-times after heptabarbital administration varied between 10 and 120 min, depending upon the dose. The time at which offset of LRR occurred could be easily determined and administration of different doses had no effect on the

Table 4. Concentrations of heptabarbital at onset and offset of loss of righting reflex, during an i.v. infusion, and after an i.v. bolus dose, respectively.

	Onset	Offset
No. of animals	7	5
Body weight (g)	188±9	195±16
Infusion time (min)	33±3	4
Total dose (mg kg^{-1})	100±8	116±9*
Duration of LRR (min)	—	100±20
Serum concn (mg L^{-1})		
Total drug	98±15	94±19
Free drug	47±7	45±6
Brain concentration (mg kg^{-1})	64±8	60±10
CSF concentration (mg L^{-1})	31±7	26±5
Protein binding in serum (%)	51±8	50±12

Results are reported as mean ± s.d.
* $P < 0 \cdot 05$ by Newman-Keuls test.

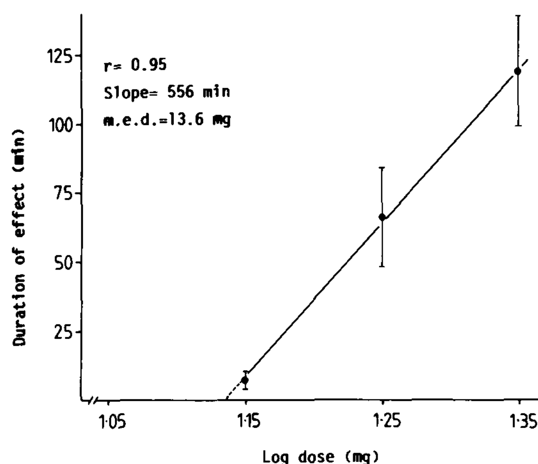

FIG. 1. Relationship between the duration of LRR and the logarithm of the administered dose of heptabarbital. Results are given as mean ± s.d. of 9 rats. M.E.D. = minimum effective dose as determined by extrapolation to the abscissa.

heptabarbital concentrations at the offset of effect (Table 3). When these concentrations were determined by HPLC, an additional peak appeared in the chromatograms, presumably a metabolite, because of the more polar character of this unknown compound compared with heptabarbital itself. The relative amounts of the metabolite could be expressed as the peak height ratios of this compound versus the internal standard. This ratio significantly increased with increasing heptabarbital dose (Table 3).

In Fig. 1 the duration of LRR is plotted versus the logarithm of the administered dose. The minimum effective dose, as calculated from the intercept on the log dose axis is $13·6 \pm 1·1$ mg. Calculation of the pharmacodynamic $t\frac{1}{2}$ gave a value of $2·8 \pm 0·2$ h, which is in very good agreement with the serum half-life (Table 1).

Onset versus offset experiment

In the experiment aimed at a direct comparison of heptabarbital concentrations measured at onset and offset of LRR, animals in the offset group regained their righting reflex after a duration of LRR of 100 ± 20 min (mean ± s.d., n = 5). The only significant difference between the two groups with respect to the drug parameters was in the total administered dose (Table 4).

Discussion

Investigation of factors which influence the concentration-pharmacological effect relationship of drugs in-vivo requires the utilization of experimental strategies that clearly differentiate between changes in the pharmacokinetics and changes in the pharmacodynamics (Dingemanse et al 1988).

Potentially complicating pharmacokinetic factors in this respect are (i) differences in the stereospecific disposition of enantiomeric drugs; (ii) changes in distribution to the site of action; (iii) formation of (inter)active metabolites; (iv) development of (acute) tolerance.

The purpose of the present study was to examine the feasibility of using indirect techniques to determine the role of (inter)active metabolites in pharmacodynamic studies in-vivo. The utilization of such indirect techniques is important because many drugs are metabolized to unknown biotransformation products. Application of indirect techniques requires, however, that also the other potentially complicating pharmacokinetic factors are taken into account. For the present investigation heptabarbital was chosen as a model drug because, unlike many other barbiturates, this is not a racemic mixture. Enantiomers of barbiturates have been shown to differ not only in their pharmacokinetic properties (Breimer & Van Rossum 1974; Van der Graaff et al 1983b), but also in their pharmacodynamic properties (Downes et al 1970; Haley & Gidley 1970; Büch et al 1973; Leeb-Lundberg et al 1980) and this seriously complicates in-vivo pharmacodynamic studies (Dingemanse et al 1988).

The drug obeyed apparent first-order kinetics with an elimination half-life of 2·8 h which enables the identification of the site of action via a differential infusion rate strategy (Danhof & Levy 1984).

The site which is pharmacokinetically indistinguishable from the site of action was identified by infusion of heptabarbital at different rates until LRR. With increasing infusion rate only a slight increase in the total serum concentration at onset of LRR was observed; for the free serum concentrations the same tendency was seen, but here the differences did not reach statistical significance. For brain and CSF concentrations no rate-dependence could be established. With regard to CSF these results are similar to the ones obtained with phenobarbital (phenobarbitone) (Danhof & Levy 1984). The lesser degree of rate-dependence of heptabarbital concentrations at onset of LRR, compared with the situation encountered with phenobarbital (Danhof & Levy 1984), is a reflection of the greater lipophilicity of the former (Yih et al 1978; Toon & Rowland 1983). The results of the infusion rate experiment lead to the preliminary conclusion that both CSF and brain tissue are pharmacokinetically indistinguishable from the site of action of heptabarbital, and that at low infusion rates this may also be the case for serum. Yet, in pharmacodynamic studies, preference has to be given to the measurement of CSF concentrations, since these are essentially *free* concentrations (Danhof & Levy 1984) and the results of plasma protein binding determination may be unreliable in certain circumstances (Chou & Levy 1984).

In pharmacodynamic investigations in-vivo one has to be aware of the potential contribution of biotransformation products to the measured response (Atkinson & Strong 1977). The major routes of heptabarbital metabolism are allylic oxidation to 3′-hydroxyheptabarbital followed by dehydrogenation to 3′-ketoheptabarbital (Gilbert et al 1974) and metabolism via an epoxide-diol pathway resulting in the formation of 5-ethylbarbituric acid as the ultimate metabolite (Vermeulen 1980). In addition to these metabolites, another yet unidentified metabolite has been found in urine, which was suggested to be 7′-hydroxyheptabarbital (Gilbert et al 1974). So far, the percentage of the administered dose of heptabarbital that can be recovered from urine remains unclear (Van der Graaff et al 1983a) and the pharmacological activity of the known metabolites has not been examined. Therefore, it cannot be excluded that some of these metabolites do indeed interfere with the pharmacological activity of

heptabarbital. This is in particular the case for the hydroxylated metabolites. It has for example been demonstrated that hydroxyamobarbital possesses intrinsic anaesthetic activity (Irrgang 1965), whereas hydroxypentobarbital has been shown to act as an antagonist of the anaesthetic effects of the parent compound (Yamamoto et al 1978). Hydroxysecobarbital on the other hand is without anaesthetic activity in doses much larger than the anaesthetic dose of secobarbital (Waddell 1965). In the present study a possible contribution of metabolites to the pharmacological effects was examined by studying the concentration-effect relationship under circumstances in which different amounts of the metabolites are formed. Heptabarbital was administered in different doses which resulted in widely differing 'sleeping-times' and the concentrations at offset of LRR were compared. In the case of the presence of (inter)active metabolites, the concentration of heptabarbital at offset of LRR may be expected to be different following administration of the different doses. In case of the presence of an active metabolite there would be a trend towards a lower concentration at offset of effect following administration of a higher dose, whereas in the situation in which a metabolite with antagonistic activity is formed the trend would be in the opposite direction. No such trend was observed in the present investigation, indicating that metabolites apparently do not interfere with the pharmacological activity of heptabarbital. This is also confirmed by a kinetic analysis of the duration of the pharmacological response in relation to the administered dose. According to Levy (1966), there is a linear relationship between the logarithm of the administered dose and the duration of the pharmacological response, the slope of this relationship being determined by the elimination rate constant. In the present study the elimination half-life of heptabarbital that could be derived on the basis of this relationship (Fig. 1) appeared to be 2·8 h, which is equal to the value obtained on the basis of the concentration vs time profile following i.v. administration (Table 1). This, together with a direct comparison of heptabarbital concentrations in CSF at onset and offset of effect (Table 4), demonstrates that metabolites formed in-vivo play no role in the pharmacodynamics of heptabarbital. From the close agreement between the values of $t_{\frac{1}{2}}$ obtained in the pharmacokinetic and the pharmacodynamic experiment, it can also be concluded that application of another assumption under which the described relationship between dose and duration of response holds, is justified, viz. the elimination of the active drug from the site of action following apparent first-order kinetics.

An issue Danhof & Levy (1984) commented upon as an advantage of using onset instead of offset of effect data is the possible development of acute functional tolerance. It has been suggested that acute tolerance to some effects of barbiturates can develop very rapidly (Maynert & Klingman 1960). According to Okamoto & Boisse (1981), tolerance only can develop for those functions which have persistently been depressed for some time during treatment. In the offset experiments that were included in the present investigation the phenomenon of development of acute pharmacodynamic tolerance may have occurred. The heptabarbital concentrations in the different compartments at offset of LRR were seen to be independent of the duration of anaesthesia, and were similar to the concentrations at onset of LRR, which points to the absence of development of acute tolerance.

The results of the present study demonstrate the appropriateness of heptabarbital as a model drug to investigate the influence of several variables on the concentration-anaesthetic effect relationship of barbiturates. Combination of the several strategies employed shows the reversibility of the pharmacodynamic measure, which is correlated directly with the heptabarbital concentration in CSF. Indirect techniques have excluded an interfering role of drug metabolites. The strategies described permit a distinction between pharmacokinetic and pharmacodynamic alterations and have been applied to evaluate the effect of experimental renal dysfunction on the concentration-effect relationship.

Acknowledgement
The authors are indebted to Prof. Dr D. D. Breimer for critically reading the manuscript.

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