EVALUATE THE PROCESS OF STEREOTAXIC INJECTION OF THE HIPPOCAMPUS BY A HOME - MADE SYRINGE

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ABSTRACT

Purpose: *We aimed to to evaluate the process of stereotaxic injection of the rat hippocampus; determined the volume of solution, and the speed of the injection of the solution into the hippocampus of the rat by a home-made syringe.*

Methods: *Experimental study, controlled description.*

Results: *The study identified the process of stereotaxic injection of the rat's hippocampus (including eight steps: immobilizing the rat to the positioning frame; exposing the skull bones to find Bredma, Lamda points; positioning the hippocampus on the skull; drilling the skull; putting the needle into the hippocampus; injecting the solution; saving the needle; pulling out the needle). The average time of anesthesia for both groups was 31.8 ± 7 minutes. The average time to perform the process of injection with a solution was 38 ± 4.07 minutes. Determine the coordinates of stereotaxic injection of the rat hippocampus (coordinates: AP = 4 mm; ML = 2.2 mm; DV = 3 mm); injection dose of 2.5 µl of solution; injection speed 1.13 µl/min. The dose and speed of the injection of the solution as above did not affect the health of the rat.*

Keywords: Hippocampus, stereotaxic injection, electric injection pump

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1. INTRODUCTIONS

Experiments on animals have been widely applied by scientists. In biomedical experiments, the rat is the most commonly used animal. Many surgical techniques and methods also have been tested by researchers in rats, including stereotaxic techniques. This is a stereotaxic approach of a target structure "Stereotaxy", which is considered "a method in neurosurgery and neuro research to locate points in the brain by an external system of reference, three-dimensional projection based on the Cartesian coordinate system" [1].

The hippocampus is an important component in the brains of humans and other vertebrates, playing an important role in the consolidation of short-term and long-term memory and navigating spatial memory. This is also one of the structures that scientists have carefully studied. According to research by Knierim J.J., since the 1957 report of the case study the patient had lost the ability to form new memories hippocampus and nearby temporal lobe structures for the treatment of intractable epilepsy [2], there have been many reports of stereotaxic injection of the rat hippocampus.

However, there have been no reports evaluating the implementation process of this technique. In addition, it is difficult to deliver very small amounts of fluid (microliter) over time into the hippocampus. In the world, some laboratories used Hamilton Syringes and Microinjection Syringe Pump systems to deliver drugs into the hippocampus. But this method was not suitable for exploratory studies. On the other hand, the speed and dosage of injected solution were not inconsistent. In Vietnam, there are currently no reports on stereotaxic injection of the rat hippocampus.

Starting from the above fact, we carried out this study to evaluate the process of stereotaxic injection of the rat hippocampus; determined the volume of solution, and the speed of the injection of the solution into the hippocampus of the rat by a home-made syringe.

2. SUBJECTS AND METHODS

2.1. Subjects

12 adult, healthy white rats, regardless of sexes. Rats were provided by the Animal Husbandry Department, Military Medical Academy according to the standards of research animals. The experiments were conducted at the Department of Practical and Experimental Surgery-Military Medical Academy, from October 15, 2022 to November 15, 2022.

2.2. Methods

- Study design: Experimental study, controlled description

- Study sample size: Determined according to the Code of regulation emphasizing the principles of "3R", using the one-way ANOVA design formula in animal studies for group comparison application [3].

Applying the formula: n = DF/k+ 1

Included:

n - The number of samples per group;

DF- Degrees of Freedom, with a minimum value of 10 and a maximum value of 20;

k- The number of study groups

- Methods of the study: 12 rats were randomly divided into 2 groups:

+ Group 1 (research group): 6 rats injected the solution to evaluate the dose and injection speed with a home-made syringe.

+ Group 2 (control group): 6 rats injected with fake injection (did not inject the solution).

- Equipment and techniques used in the study:

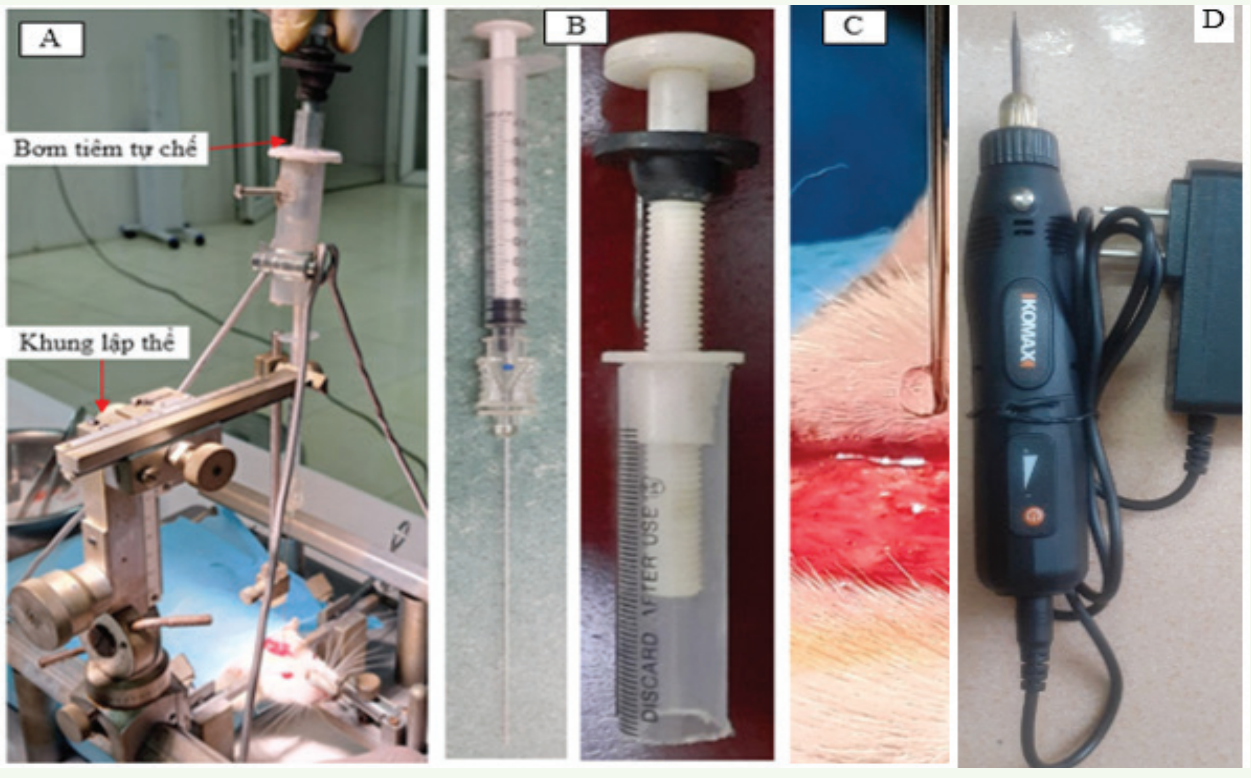

Figure 1. The equipment used: A) stereotaxic frame and home-made syringe; B) 25G needle attached to a 1 ml syringe (left), the force transfer unit of the home- made syringe pump (right); C) 01 drop of NaCl on the tip of the needle when rotating for 15 minutes; D) Komax mini drill

+ The stereotaxic framework used in this study is the Narishige (Japan) type SR-6, number 9009.

+ Home-made syringe has 2 main parts:

(1) The injector is a 1 ml syringe with a 25G needle (taken from a spinal needle), the needle had been cut off the beveled tip and smoothed the top of the needle;

(2) Force transfer parts, which convert from rotational force to thrust force to adjust the pumping force, thereby, adjusting the volume of the pumped solution.

Home-made syringe structure included: 01 syringe of 5 ml; 01 spiral thread that can rotate the inner thrust shaft; 01 circular divider (clock shape) located on the horizontal axis of the syringe, on the top of the thrust shaft attach a small metal bar as a pointer on the clock face. The syringe operated on a rotating mechanism to adjust the force exerted on the piston of the 1 ml syringe to inject slowly and deliver 2.5 µl of solution (0.9% NaCl solution) to the needle tip when rotated for 15 minutes.

+ Mini drill and cranial drill of Komax (Germany).

- Determine the volume of solution pumped out of the needle tip: take 0.1 ml of 0.9% NaCl into a 1 ml syringe, and rotate the force transfer unit clockwise, every 15 minutes, see 01 drops of solution flow to the tip of the needle, until 0.1 ml of the solution has 40 drops. Each drop contains 2.5 µl of 0.9% NaCl.

- The technique of stereotaxic injection of the hippocampus was performed according to the process of Jean Y.Y et al (2015) [9].

+ Preparation before surgery: Sterile surgical instruments, electric syringes, drugs, chemicals.

+ Prepare animals: Weigh the rat, and anesthetize with ketamine peritoneal injection (dose 1 mg/kg). Monitor depth of anesthesia due to loss of toe reflex.

+ Fix the rat to the navigation system with the incisor bar and the horizontal bar behind the auditory hole, and apply tobradex ointment to the eye to keep it moist.

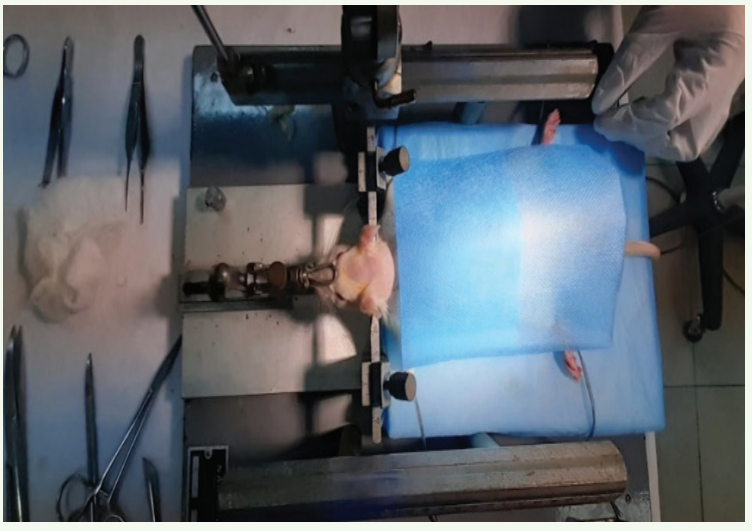

Figure 2. Fixing the rat to the navigation

+ Exposing the skull: Disinfect the skin on the rat's skull with a solution of betadin and 70% ethanol respectively. Make an incision of 2-3 mm in the center of the scalp. Make an incision of 2-3 mm in the center of the scalp. Use straight scissors to extend the incision to 1.0-1.5 cm, exposing the skull. Determine the Bregma and Lambda points and mark with a sterile fine dot pen. Adjust the Lambda point and the Bregma point to lie on the same horizontal plane.

+ Determine the coordinates of the hippocampus on the skull according to the rat brain atlas [4] and according to the study of Wang D.L [5] (AP = 4 mm, ML = 2.2 mm, DV = 3 mm). Return the coordinates on the Stereotaxic system to position (0, 0, 0). Return the tip of the small metal rod to the Bregma point position. Determine and mark the ML coordinates (adjust the microlevelers to the coordinates AP = 4.0 mm; ML = ± 2.2 mm).

+ Drill the skull: Put the drill head 0f 0.8 mm to the drill bit and perform drilling with both hands.

+ Hippocampus injection: Attach 25G syringe (prepared) to the positioning system, expel air from the syringe by rotating for 15 minutes the force transfer of the home-made syringe (push 2.5 µl of fluid out of the needle tip). Return the needle tip to one of the ML holes just drilled; put the needle to the coordinate DV = - 3.0 mm. Slowly rotate (within 15 minutes) the force transfer of the home-made syringe to push 2.5 µl of solution to the tip of the needle; Save the needle for two minutes (for the control group, only put the needle down to the hippocampus coordinates but did not inject the solution). Pull out the syringe and inject antibiotics into the cranial cavity. Move the needle to the other side of the brain and repeat the steps. Sew the rat's scalp. Remove the rat from the equipment and provide post-operative care.

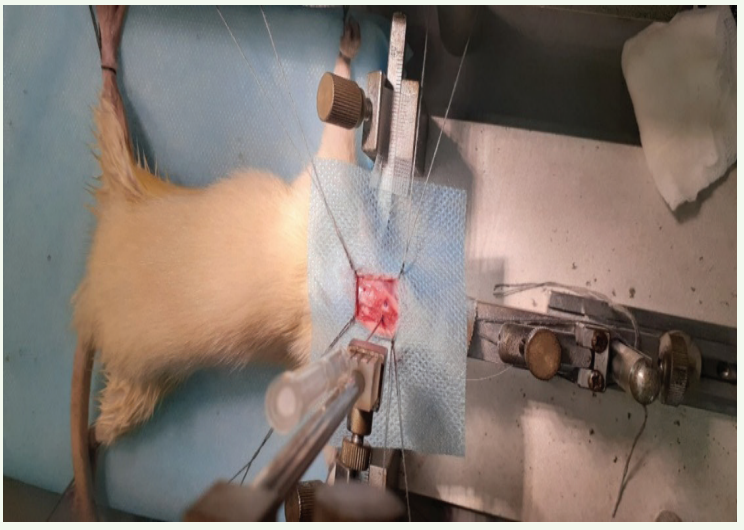

Figure 3. Exposing Bregma and Lambda points. Adjust the points Bregma and Lamda on the same horizontal plane, determine the coordinates of the hippocampus on the skull

- Study ethics: The study topic was approved by the Ethics Committee of Biomedical research No. IRB-A-2201.

- Data processing: Using Excel software SPSS 20.0. Calculate the mean value with the quantitative variable, calculate the percentage with the qualitative variable, compare the mean with the T tests, compare the percentage with the Chi-square test. The difference was statistically significant when $p < 0.05$.

3. RESULTS

3.1. General condition of rats before and after intervention

Table 1. Rat weight before and after the intervention two weeks

Rat group		Rat weight (± SD)	p
Group 1 (n = 6)	Before intervention	196.7 ± 25.8g	> 0.05
	After intervention	182.5 ± 30.6g	
Group 2 (n = 6)	Before intervention	195.0 ± 18.7g	> 0.05
	After intervention	194.2 ± 16.0g	
Total (n = 12)	Before intervention	195.8 ± 21.2g	> 0.05
	After intervention	188.3 ± 24.0g	

Before the intervention, each rat in group 1 had an average weight of 196.7 ± 25.8g (lowest 160g, highest 230g), each rat in group 2 had an average weight of 195.0 ± 18.7g (lowest 170g, highest 220g), each rat in both groups had an average weight of 195.8 ± 21.2g. There was no difference in rat weight between group 1 and group 2 ($p > 0.05$). In both groups of rat, there was no difference in weight before and after the intervention two weeks ($p > 0.05$).

Table 2. Rat status after two weeks of intervention

Variable	Group 1 (n = 6)	Group 2 (n = 6)	Total (n = 12)	p_{1-2}
Normal movement	6 (100%)	6 (100%)	12 (100%)	
Stimulation	2 (30.3%)	1 (16.7%)	3 (25%)	0.55
Ruffle feathers	0	0	0	
Live rat	6 (100%)	6 (100%)	12 (100%)	

There 100% of the rats in the study had normal movement, no ruffle feathers expression, 100% of the rat lived, 25% of the rat showed arousal expression when feeding (in which, group 1 was 30.3%, group 2 was 16.7%), there was no difference in arousal status between the two groups of rat (p = 0.55).

3.2. The results of stereotaxic injection of rat hippocampus

Table 3. Time of insensitivity

Variable	Group 1 (n = 6)	Group 2 (n = 6)	Total (n = 12)
Time of anesthesia (± SD)	30.0 ± 7.1 min	33.7 ± 7.0 min	31.8 ± 7.0 min
p	> 0.05		

The average anesthesia time of rats in group 1 (30 ± 7.1 minutes) and rats in group 2 (33.7 ± 7 minutes) there was no difference, with p > 0.05. Calculating the total number of studied rats in both groups, the anesthesia time of rats was from 20-45 minutes, the average was 31.8 ± 7 minutes.

Table 4. Time to perform stereotaxic injection of the rat hippocampus

Variable (± SD)	Group 1 (n = 6)	Group 2 (n = 6)	Total (n = 12)	p
Time to fix the rat on the positioning frame (minutes)	11.17 ± 2.64	12.5 ± 1.87	11.83 ± 2.29	> 0.05
Time of exposing the skull (minutes)	5.5 ± 1.04	4.67 ± 1.63	5.08 ± 1.38	> 0.05
Time to locate hippocampus (minutes)	12.33 ± 1.75	13.17 ± 1.47	12.75 ± 1.6	> 0.05
Drilling time (minutes)	1.33 ± 0.41	1.42 ± 0.49	1.38 ± 0.43	> 0.05
Time of needle putting to the hippocampus (minutes)	1.75 ± 0.27	2.0 ± 0.32	1.88 ± 0.31	> 0.05
Injection time (minutes)	1.58 ± 0.58			
Injection speed (µl/ minutes)	1.13 ± 0.39			
Retention time (minutes)	2.0		2.0	
Time of needle pulling out (minutes)	2.33 ± 0.61	2.58 ± 0.38	2.46 ± 0.49	> 0.05
Total time to perform the process without injection (minutes)	34.42 ± 4.0	36.33 ± 2.54	35.38 ± 3.35	> 0.05
Total time to perform the process with injection (minutes)	38.0 ± 4.07			

The results of study showed that, there was no difference between the two groups of rats on the time of rat immobilization in the stereotaxic frame; skull bones exposing; hippocampus positioning; drilling perform; puting down the needle to the hippocampus and withdraw the needle (p > 0.05). The time of hippocampal injection (with solution pump) in group 1 was from 33-43 minutes, average 38 ± 4.07 minutes.

- In both experimental groups of rat, the time of immobilization of rat in the stereotaxic frame from 8-15 minutes (mean 11.83 ± 2.29 minutes); exposing

the skull to find Lamda and Bregma points from 3-7 minutes (mean 5.08 ± 1.38 minutes); locating the hippocampus on the skull from 10-15 minutes (mean 12.75 ± 1.6 minutes); skull drilling from 1-2 minutes (mean 1.38 ± 0.43 minutes); putting down the needle to the hippocampus for 1.5-2.5 minutes (mean 1.88 ± 0.31 minutes). Total time to perform process of hippocampus stereotaxic injection (without injection) was from 29.5-40.5 minutes (mean 35.38 ± 3.35 minutes).

4. DISCUSSIONS

In this study, our 12 experimental rats had an average weight of 195.8 ± 21.2g, which was not significantly smaller than Wistar male rats in other studies (250 ± 30) [5]. We randomly divided the research rats into two groups (each group of 6 rats). There was no difference in weight (p = 0.9). 100% of the rats participating in the study showed normal conditions of movement and eating. The rats in group 1 were injected with 0.9% NaCl solution into the hippocampus. The rats in group 2 with fake injections (putting down the needle to the hippocampus and did not inject the solution).

- Regarding anesthesia: there were many methods of anesthesia for rat stereotaxic surgery, such as the use of injections, injection routes, and drug combinations. Various injectable drugs had been used as rat anesthetics for stereotaxic surgery, most commonly pentobarbital or thiopental. These were drugs with proven sedative effects but were not reliable anesthetics when used alone [6]. Therefore, the combination of injectable anesthetics provides the best anesthetic effect, such as the combination of fentanyl-droperidol and ketamine-medetomidine or xylazine. Ketamine-xylazine was one of the most common anesthetic combinations in mice, rats, hamsters, and guinea pigs.

- Ketamine is an N-methyl d-aspartate antagonist, with anesthetic and analgesic properties in many species. Besides analgesic and anesthetic properties, ketamine has many advantages such as simple to use, wide safety, and the ability to combine with other drugs. Xylazine is an α2-adrenoreceptor agonist, with sedative effects, mainly used for sedation and analgesia. Ketamine and xylazine are both rapidly absorbed and well distributed to the Central Nervous System. Both drugs are metabolized mainly by cytochromes P450 enzymes in the liver and excreted by the kidneys. In this study, we used ketamine (1 mg/kg) for anesthesia through peritoneal injection, the average time to achieve anesthesia was 31.8 minutes (shortest 20 minutes, longest 45 minutes); longer than the duration of anesthesia with the combination ketamine - xylazine (average 30 minutes) [6].

- The process of hippocampus stereotaxic injection: after anesthesia (rats lose the reflex of legs contraction when painful stimulation), immobilize rat to the frame and perform hippocampus stereotactic injections. During the process from skull drilling (constantly spraying physiological saline to reduce heat at the drill site), putting down the needle to the hippocampus, injecting and pulling out the needle (performed under a 10X microscope), rats were monitored breathing and abnormal movements.

If detected the rats with abnormal signs (breathing faster or slower, ruffle feathers, muscle contraction, etc.), stop the technique and continue only when the rat was stable. The results of study showed that, the average total time of stereotaxic injection of the rat hippocampus in group 1 was 38 ± 4.07 minutes, shorter than the time of process of stereotaxic surgery of Cheng S (one hour) [8]. This difference may be in Chen S's study, the canula was implanted in the bilateral hippocampus, then injected.

- The average time for each step in the process of 12 rats was:

+ Fix the rat to the stereotaxic frame: 11.83 ± 2.29 minutes. In this step, it was necessary to use the pliers for rat mouth opening to fix the two front teeth to the incisor bar on the positioning frame. Then, adjust the two ear bars to fix the two sides of the rat ear cavity to the stereotaxic frame, make sure the rat head was in the center by adjusting the coordinates of the ear bar along the side axis on each side to be equal and equal to 21.5 mm.

+ Expose rat skull, find Lamda and Bregma points: 5.08 ±1.38 minutes

+ Determine coordinates of the hippocampus on rat skull: 12.75 ± 1.6 minutes. A very important operation was to ensure that the two points Bregma and Lamda lie on the same plane by adjusting the incisor bar up and down to determine the dorsal-abdominal coordinates of the Lamda and Bregma points. In our study, this 2-point coordinate difference was about 0.03 mm (consistent with the study of Cheng S et al.).

+ Skull drilling: Average was 1.38 ± 0.43 minutes

+ Putting down the needle to the hippocampus: 1.88 ± 0.31 minutes

+ Injecting solution into hippocampus (only performed in rats of research group): 1.58 minutes

+ Save needle: 2.00 minutes. The time of needle retention was to minimize backflow of solution from

the injection site. This time was different in studies, such as the study of Jean Y.Y in 2015 (Save needle -one minute) [9], Wang D.L 2004 (Save needle: five minutes) [5], Leonard E in 1989 (Save needle: five-ten seconds) [10], and Moser M.B in 1998 (Save needle: two minutes) [11]

+ Pull out the needle: 2.46 ± 0.49 minutes

Several studies in modern laboratories in the world on the use of microsyringe system in hippocampus stereotaxic injection ensured accurate dose and injection speed. The authors usually used the amount of fluid injected into the hippocampus from 0.1-3.0 µl, the injection speed was 0.1-0.5 µl/min [5], [7]. In this study, we injected 2.5 µl of 0.9% NaCl solution with the speed of 1.13 µl/min. Follow-up after two weeks of intervention, found that 100% of rats were alive, there was no sign of ruffle feathers.

However, there were 25% of rats with signs of stimulation when feeding, in which, the group with NaCl injection was 30.3%, the group with fake injection was 16.7%, there was no difference in stimulation status between the two groups, with p = 0.55. The stimulation status disappeared after two days of intervention. Possibly this was a transient symptom, so it was not mentioned in the stereotaxic injection studies of the rat hippocampus. We also did not find any difference in weight between group 1 and group 2, both before and two weeks after the intervention (p > 0.05).

Thus, with a dose of 2.5 µl and injection speed of 1.13 µl/min of 0.9% NaCl solution as above, the process of stereotaxic injection of the rat hippocampus by Home-made syringe in the study did not affect to overall rat health.

5. CONCLUSIONS

An experimental, controlled and descriptive study on 12 rats with stereotaxic injection of the rat hippocampus by a Home-made syringe (6 rats in the research group and 6 rats in the control group), we concluded:

- Determine the process of stereotaxic injection of the rat hippocampus by Home-made syringe, including eight steps:

(1) Fixing the rat to the positioning frame;

(2) Exposing the skull to find Bredma, Lamda points;

(3) Locating the hippocampus on the skull;

(4) Drilling skull;

(5) Putting down the needle to the hippocampus;

(6) Injecting solution;

(7) Saving the needle;

(8) Pulling out the needle.

- The average time of insensitivity was 31.8 ± 7.0 minutes; the time to perform the process of stereotaxic injection of rat hippocampus with Home-made syringe (with solution) was 38.0 ± 4.07 minutes.

- Determine the injection coordinates of the rat hippocampus: AP = 4 mm, ML = 2.2 mm, DV = 3 mm; with an injection dose of 2.5 µl of solution and an injection speed of 1.13 µl/min.

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