Fluid Nanocarrier for Insulin for Noninvasive Treatment of Diabetes Mellitus

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Research

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Abstract

Background

A real solution for diabetics to end their suffering with injection and the consequences of poor patient compliance based on the understanding how the body releases insulin as well as the nature of insulin is an argent demand. In this study a novel management strategy was developed using fluid nanocarrier as well as a solution of insulin to treat this problem. The developed nanocarrier is a microemulsion (ME) containing insulin. The transdermal flux of insulin was estimated through rat's skin using a Franz diffusion cell. Moreover, the efficacy of the treatments was assessed orally and transdermally in rats.

Results

Based on the rheological properties and droplets size results the formulated fluids were microemulsions. Also, a flux of insulin as high as 1.77±0.22 iu.cm^{-2}.h^{-1} through rat's epidermis could be achieved. The short term monitoring of blood sugar level after transdermal application exhibited a slight decrease. On the other hand, the frequent application could achieve a satisfied decline. However, the rapid and significant reduction of the blood sugar level after oral application was surprised. The X-raying of the GI after oral application of the preparation showed high illumination in the lower part of the esophagus and upper part of the stomach.

Conclusion

This study reveals high potential esophageal absorption using fluid dosage forms. It must be taken in consideration as sublingual and intranasal application. The developed nanofluid can control the blood sugar level orally or in combination with transdermal application and help the diabetics to adhere their therapeutic course.

Introduction

Diabetes is a metabolic disorder, caused by low sensitivity to insulin (type 2) or fail or decrease of its productivity (type 1). It is identified by high blood sugar level [1–2]. A replacement therapy using insulin is used for the treatment of insulin-dependent diabetes mellitus or type-I diabetes [3]. Insulin is a biomolecule, synthesized in the body in pancreas. It is composed of 51 amino acid with a molecular weight about 5.7 kDa. It contains 2 peptide chains: Chain A (21 amino acid residues) and chain B (30 amino acid residues) [4]. The protein nature, high molecular weight, hydrophobicity of insulin makes its stability and absorption through oral route not granted. Hence, it is subcutaneously or by intravenous route delivered [5].The complexation of insulin with zinc in the presence of acetate buffer results in an amorphous precipitate or a crystalline composite depending on the pH of the acetate buffer solution. Depending on their solubility, they are combined commercially to produce rapid or slow absorption (6). Injection of insulin is painful. This becomes harder that it must be applied daily and for lifelong which

can cause a poor dug compliance and in its turn can lead to serious complications [7–8]. Hence many researchers attempted to find other routes of administration [9].

The idea of overcoming the stratum corneum to facilitate the transdermal penetration of insulin attracted many scientists [10–13]. Some of them preferred to pore it using micro-needles (10). Others tried to facilitate the transdermal penetration with aid of electroporation [14], sonophoresis [15] or iontophoresis [16]. The nasal and sublingual routes also were under investigation [17–18]. Many other scientists preferred the nanoparticles approach to overcome the gastric juice, enzymatic digestion and lipophilicicty of cell membranes to pass through gut wall into the blood circulation [19–21].

Microemulsions (MEs) are fluid, easily produced, easily release the drug with high penetration potential in comparison to nanoprticles. Furthermore, they can be administer orally and transdermally [22–24].The biological activity of insulin was reported in an early study for formulated insulin in a double emulsion after oral application [25]. On the other hand, the using of microemulsions as drug delivery system for insulin either orally, transdermally, nasally and sublingually were assessed by many researchers [17–18, 26–27]. However, the type of the microemulsion (W/O or O/W) regarding insulin encapsulation, the used components and the final efficacy in the preparation and their toxicity are in question.

This study aimed to develop a new fluid formulation flexible for dosing and route of administration for both oral and transdermal application and pharmaceutically acceptable to simulate the release of insulin in the body.

Methods And Instruments

Injectable insulin solution (Actrapid® from Novo Nordisk, Demark) was purchased from a local pharmacy. Anhydrous sodium sulfate was purchased from Sigma-Aldrich (Steinheim, Germany). Insulin powder was purchased from Sigma (Denmark). Streptozotocin was purchased from Bioworld OH, USA. Sorbitanmonolaurate (Span® 20) and Poloxyethylenesorbitan.mono-oleate (Tween® 80) were purchased from SIGMA, France. Magnesium Chloride was purchased from SD.Fine Chem Limited (India). Methanol HPLC grade was purchased from Fulltime (Anqing, China). Water for HPLC was purchased from LabChem (Zelienople, USA). Dimethyl sulfoxide (DMSO) and Isopropyl Myristate (IPM) were obtained from Merck (Darmstadt, Germany).

High pressure chromatography (HPLC)-method

The concentration of insulin was estimated via Thermo scientific, Dionex Ultimate 3000 HPLC chromatographic system made in Germany connected with diode array detector. The estimation was performed by injecting a volume of 20 µl from the samples into C18 column (4.6*250 mm) then separating using a mobile phase composed of 25% acetonitrile and 75% of anhydrous sodium sulfate solution pH 2.3 at flow rate of 1 ml/min at temperature of 40 ^{o}C. The solution was maintained at temperature above 25 ^{o}C during mixing with acetonitrile to prevent any precipitation. The later solution was prepared by dissolving 28.4 g of anhydrous sodium sulfate, adding 2.7 ml of phosphoric acid and

completing the volume to 1000 in a volumetric glass flask. The detection was carried out at wave length of 214 nm.

Two series of insulin were prepared for establishing the calibration curve of insulin. First series was prepared by dissolving insulin powder in 0.01 M HCl solution then preparing standard with concentration between 0.1-10 iu/ml (Efficacy: 1 mg contains 27.5 iu). The second one was for injectable insulin solution flacon by diluting it to similar concentrations.

Preparing of DMSO Solution

The solution prepared by adding 1.8 ml DMSO gradually to a beaker containing 1ml insulin solution. The mixture was kept cooled during the addition by putting the beaker in a cooled water (4 ^{o}C) that the process here is exothermic.

Producing of microemulsions

Insulin and DMSO were mixed first together in a beaker then isopropyl myristate was added. The rising of the temperature during the mixing of insulin solution and DMSO was avoided by adding DMSO gradually and cooling. The surfactants were added drop wise into the beaker with continuous stirring over a magnetic stirrer until forming a clear microemulsion.

Viscosity measurements:

The viscosity and rheological properties of MEs were measured at 25 ^{o}C using an electric Rheometer (model MCR 302) made by Anton Paar in Germany. It is used to establish the MEs using bob and cup technique.

Droplet size measurement (Zeta-potential measurement)

A Zeta-sizer (Nano series HT) made by Malvern in USA was used to determine the zeta average and the poly dipersity index (PDI).

Preparing rat's skin

All the procedures were performed according to the NIH Guidelines for the Care and Use of Laboratory Animals and which is approved by the Animal Ethics Committee of the University. The rats (Wistar male rats, 220 – 250 g, 10 –12 weeks old) were shaved carefully then anesthetized using ethyl ether before executing. Then the skin was peeled and cleaned from adipose tissues carefully. The skin was cut to small pieces to fit with Franz diffusion cell (a bit diameter larger than 15 mm) then stored in a freezer at a temperature of -70 ^{o}C.

Peeling of the epidermis layer

Frozen rat's skin pieces were soaked in the 2M solution of magnesium chloride at 4 ^{o}C overnight. The epidermis layer were carefully peeled using a forceps. The integrity of the epidermis was tested against the light after fixing it on orifice of donor compartment (This process is done in a large beaker filled with phosphate buffer). The upper surface of epidermis must be toward the donor compartment and the lower surface toward the accepter compartment.

Transdermal Study of Insulin using Franz cell

For studying the transdermal absorption of insulin through rat's skin, a single Franz diffusion cell mad by Hanson in USA (15mm diameter, 7ml acceptor volume) was used. The cell was kept at temperature of 32 ± 1 ^{o}C during the test by connecting it with a thermo circulator water bath. The acceptor compartment was filled with 7 ml of phosphate buffer. Only 0.1 ml of each preparation was allocated over the epidermis. A flange was used to fix a glass disc and the ring over the donor compartment with acceptor compartment. Half ml was removed after 0.5, 1.5, 3.5, 5.5, 6.5 and 24 h for analyzing the penetrated drug through the epidermis using HPLC-method. The removed samples were replaced by equal volumes of the same acceptor medium.

Inducing diabetes in rats using streptozotocin

Streptozotocin was dissolved in citrate buffer 4.5 pH at concentration of 20 mg/ml under laminar flow condition. The citrate buffer was prepared by dissolving 1.49 g citrate and 1 g citric acid in 100ml water. The streptozotocin solution was prepared directly before using. A volume of streptozotocin solution (calculated for an amounts 50 mg/kg (eq1)) was injected intraperitoneal to a group of overnight fasted rats. The rats were fed after injection directly till evening then the food was removed and the rats fasted again. The blood sugar level was measured next day at mooring. The rats which showed blood sugar level higher than 250 mg/dl considered to be diabetics.

$$V\ (ml) = \frac{50 \times wt(kg)}{20} \quad \text{eq.1}$$

Efficacy of insulin after transdermal application in rats based on response

An adequate volumes of each of the DMSO solution, microemulsion (MEIn2) and aqueous solution of insulin were allocated over 4 cm^2 shaved area on the back of 4 white male rats for each preparation at morning at dosing level of 0.9 iu/kg of insulin. The effect of insulin was monitored over six and half hours after application of insulin. Also, the blood sugar level was measured after transdermal application of insulin every two days.

Efficacy of insulin after oral application in diabetic rats

The DMSO solution and the microemulsion (MEIn2) were injected orally at dosing level of 0.9 iu/kg of insulin into 3 white male rats for testing each preparation. The blood sugar level was monitored after 0.5,

1, 1.5 and 3h.

Sugar blood level test

The evaluation of blood sugar level was performed using Blood-Glucose Meter ACCU-CHEK® Performa from Germany. A one blood drop, which was obtained by bleeding Retro-Orbital Plexus was used for measuring blood sugar level of 12 h fasted rats at morning.

X-ray–measurement

The measurements were performed by injection 0.5 ml of the microemulsion. A 0.5 ml of iopromide (370mg/ml) (Ultravist-370 from Bayer Germany) was used as contrast agent to prepare the microemulsion to make the preparation detectable in the gastrointestinal tract by X-ray. The rats were anesthetized with aid of a beaker containing a wick of cotton wetted with ethyl ether before X-raying them. Ecoray Xray made in Korea was used for X-raying the rats after 0.5, 2, 3.5 and 6.5 h after the oral injection.

Results

Microemulsions characterization

Rheology of MEs

The rheograms of different formulated MEs (Figure 1) were established by measuring each of the viscosity and shear stress against the shear rate at temperature of 25 ^{o}C.

Figure 1: Rheograms of prepared microemulsions by plotting the viscosity and shear stress against the shear rate.

Figure 1 shows that the viscosity is constant and relatively low. Also, the relationship between the shear rate and the shear stress is linear. Hence viscosity of developed MEs are Newtonian fluids.

Droplet size of MEs

The MEs were measured using zeta sizer (Figure 2) without any dilution nor filtration at temperature of 25 ^{o}C. The mean and standard deviation of the three measurements for each sample and their composition are represented in table 1.

Figure 2: Droplet size distribution of MEIn2 using zeta sizer

Table1: The composition of different developed MEs and their droplets size and PDIs

	Insulin (ml)	DMSO (ml)	IPM (ml)	Zeta-avarage	PDI
MEIn1	1	0.5	2.5	15.30±1.7	0.886
MEIn2	0.5	0.45	2.5	100.6±28.3	0.4145
MEIn3	0.5	-	2.5	308.8±120.1	0.322

The droplets sizes of the three microemulsions were 16.3, 100.6 and 308.8 nm. Hence, these formulated systems are MEs.

HPLC-method:

The HPLC-chromatogram showed a higher retention time of 14.3 min (Figure 3A) for prepared insulin standard from powder in comparison to insulin from solution for injection which showed a retention time of 9.1 min (Figure 3B). However, the linearity of the calibration curves were 99.43 and 99.93 for insulin prepared from powder and for insulin of solution for injection respectively. The expected concretions for detection in Franz diffusion cell were within the concentrations of the calibration curve. However, the ratio of high of the peak of lowest concentration (0.1 iu/ml) to the noise was much higher than 9 [28]. The recovery of insulin from our preparations was 98 ± 2.5.

Figure 3: Chromatogram of insulin of A: insulin solution of rapid injection solution; B: prepared insulin solution using 0.01M HCl.

The flux of insulin using Franz diffusion cell

The stratum corneum in epidermis is the main barrier for hydrophilic drugs therefore it is enough to evaluate the permeability through the epidermis instead of full skin for hydrophilic drugs [29]. The transdermal of insulin was evaluated by calculating the flux through isolated epidermis for DMSO insulin solution as well as for two microemulsions (MEIn1 and MEIn2) of three developed loaded insulin microemulsions. The cumulative penetrated insulin amounts per iu/hr*cm^2 are represented against the time in figure 4.

Figure 4: Cumulative transdermally penetrated amounts of insulin over 5 h using DMSO solution plus two different prepared microemulsions (MEIn1 and MEIn2)

The transdermal of Insulin through the epidermis was rapidly and could maintain the steady state only for 5 hr. The recommended applied amount was a bit little (100 µl) and couldn't keep the steady state for long time (29). However, the flux was calculated from steady state period and the results was as flowing: 0.95 ±0.12, 1.77±0.22 and 0.43±0.04 from DMSO solution, MEIn2 and MEIn3 respectively. The MEs contains DMSO showed higher flux for insulin in comparison to the flux of insulin using DMSO solution. On the other hand, insulin had higher flux via the DMSO solution in comparison to MEs free DMSO. Hence, MEIn2 was used for further studying of in vivo transdermal and oral bioavailability.

The transdermal efficacy of loaded insulin microemulsions in rats:

First the response of insulin was monitored by measuring the blood sugar level over six hours after application of insulin in MEln2, DMSO solution and water solution over the skin of 4shaved 12 h fasted rats and the results are represented in figure5.

Figure 5: Blood sugar level during 6:30 h in rats (7-18) after transdermal application of insulin using DMSO solution (SR 7-10), MEln2 (MER 11-14) and water solution of insulin (WR 15-18)

The DMSO solution and the MEln2 containing insulin caused a slight decrease of blood sugar level where the water solution of insulin didn't cause a remarkable decease of blood sugar level. When the test was repeated after two days, it was observed that the initial blood sugar level before the study was in lowering. Therefore, the formulations were applied in interval two days and the blood sugar level measured next day after application. These results are represented in figure 6

Figure 6: Blood sugar level during 2weeks in rats (7-18) after transdermal application of insulin every 2 days using DMSO solution (SR 7-10), MEln2 (MER 11-14) and water solution (WR 15-18)

Figure 6 shows no instantaneous lowering for blood sugar but gradually during the treatment over two weeks. Also, the significant decrease was recorded after 6 days using the DMSO solution where was relatively faster using MEln2 which was recorded after 4 days. However, no significant change was noted of blood sugar level after using the aqueous solution of insulin.

Efficacy of oral insulin loaded microemulsions

The blood sugar level was monitored over 3 hours after oral application of insulin and the response against the time are represented in figure 7.

Figure 7: Blood sugar level during 3 h is rats (1-6) after oral application of insulin using DMSO solution (Solution R1-3), MEln2 (Microemulsion R4-6) and water solution (WR)

Figure 7 shows a rapid effect after 30 min of application of insulin orally. The lowering was dependent on the initial sugar level on both of preparations.

Xray measurement

Flowing the ME by X-ray in GI showed that the drug after 15 min is still in the stomach (Figure 8). However, the lower part of the esophagus, sphincter and upper region of the stomach were highly illuminated. Which may refers to primary absorption in this region. However the illumination was detected in the small intestine after 6:30h.

Figure 8: Xray images of orlally injected 0.5 ml ME contianing of iopromide as a contrast agent.

Discussion

Normal blood insulin level increases many time after a meal. Also, insulin dose varies broadly between diabetics depending on the patient status. These conditions make the formulation of insulin in a solid dosage form at a fixed dose difficult. Furthermore, the applying insulin in a sustained manner such as by different transdermal approaches can mimic the fasting insulin secretion but will not mimic the secretion after meals [30–31]. Applying of insulin in a fluid dosage form provides a reasonable deal to help the diabetics to tolerate the disorder. Using fluid dosage form for oral application, the dose can be easily varied among patients in contrast to solid dosage form. Even, by varying application area over the skin by transdermal application, the flux of insulin varies though the skin. Hence, the individualization of the dose of insulin in a fluid is possible. The oral absorption is rapid in contrast to the transdermal application which rises the plasma level slowly and in controlled manner [32]. Hence, a combination of both routes can help to control blood sugar level in similar way to pancreatic secretion or a mixture of crystal and amorphous insulin [4,6].

A hydrophilic, high molecular weight molecule such as insulin has limited oral absorption. Furthermore, its protein nature limits the stability by enzymatic degradation. Additionally, the stratum corneum is a barrier for hydrophilic and high molecular weight drugs [5, 33–34].

Many reported techniques for improving the transdermal application have limitations [10–16]. The microneedles patches which based on a damage of the epidermis, can potentiates unhealable wounds by recurring application [35–37] where other approaches increase the treatment cost and need some skills through using special instruments. On the other hand, the nanoparticles which are not easily prepared and should applied in solid dosage form at the end which makes the individualization the dose difficult [19–21].

In this study, the technique of fluid microemulsion is used to overcome the different previous mentioned limitations. The encapsulation of insulin fluid micelles can protect insulin from degradation as well as facilitate the absorption through the skin and the gut wall beside to individualize the dose [38–39].

The characterization results showed that the formulated systems of insulin are fluid MEs. The expected encapsulation of insulin is 100% in the MEs that insulin is hydrophilic drug and the produced MEs are W/O [40].

Despite insulin is hydrophilic molecule and has high molecular weight, the transdermal study using Franz diffusion cell showed that it can pass through the epidermis using the DMSO solution as well as when encapsulated in the MEs. The flux of a drug though the skin is by passive diffusion. Hence it can be improved by increasing the surface area and by increasing the concentration. These facts can be used to adjust the dose at a site of application by varying the area and amount depending on the required dose [41–42].

The results of monitoring blood sugar level for 6:30 h showed that low efficacy of the transdermal applied formulations that penetrated amount was not sufficient to induce a significant pharmacologic effect. However, the significant response which was achieved by repeating the application may be related

the resulted accumulation from repeating application as the skin behaved as a reservoir. Hence, a stronger therapeutic effect can be achieved either by repeating the application especially if the dosing interval was decreased or by increasing the applied concentration.

The rapid response after oral application indicates that the drug may be absorbed from the mucosa of upper part of the GI behind the mouth. The X-ray images indicates to an absorption at lower part of the esophagus and the upper region of the stomach. This study highlights a new important area for drug absorption for fluid dosage forms can be targeted to gain a rapid absorption without degradation especially for biologicals. This site of application can be added for investigation such as in the sublingual, intranasal and buccal application. The anatomic surface area of esophagus is 235 cm^2 which is approximately the half surface area of the stomach (500 cm^2) and larger than of the oral cavity (197 cm^2), the sublingual (26.5 cm^2) and the buccal (50.2 cm^2) surface areas [43–44].

The toxicity of used ingredient especially the surfactants are of the main limitations of using the microemulsions [45]. However, the used nonionic surfactants in this study are nontoxic and can be used even as food additives [46–47]. Besides, FDA classifies DMSO in class3 as ethanol in the safest solvent class [48]. On the other hand, the rats did not show any toxic symptoms or skin redness after the treatment.

Conclusion:

The new developed containing insulin systems are nontoxic fluid microemulsions. They enable the delivery of insulin through the skin as well as orally. Using them it is possible to individualize the dose and control the blood sugar level by diabetics. The fluid dosage forms can deliver rapidly by primary absorption form the mucosa of the esophagus.

Abbreviations

DMSO
dimethyl sulfoxide
h
hour
HPLC
High pressure chromatography
In
insulin
IPM
Isopropyl Myristate
ME
Microemulsion
PID

poly dispersity index
R
rat
S
DMSO solution
W
water

Declarations

Conflict of interest:

The author declare no conflict of interest.

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Figures

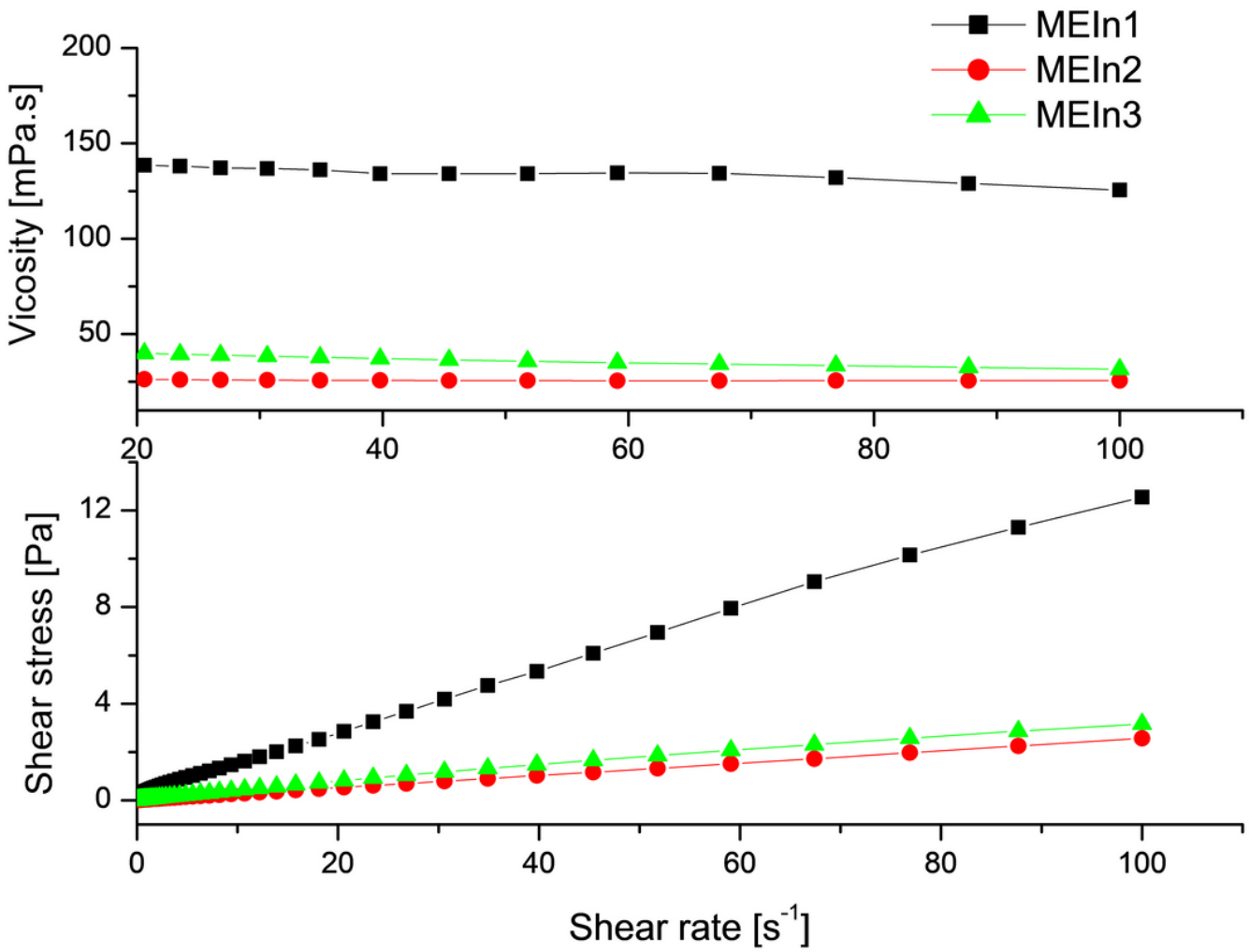
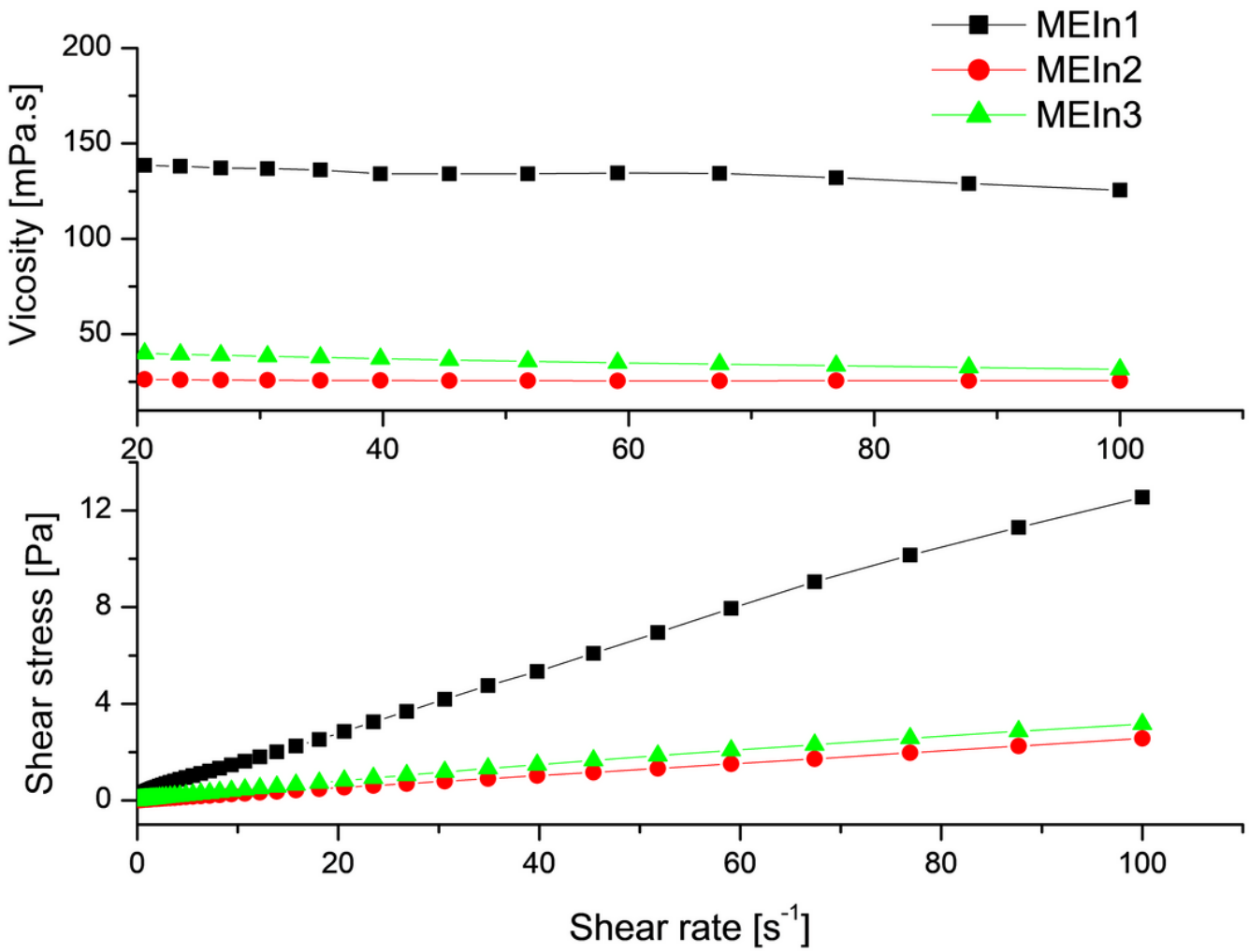


Figure 1

Rheograms of prepared microemulsions by plotting the viscosity and shear stress against the shear rate.



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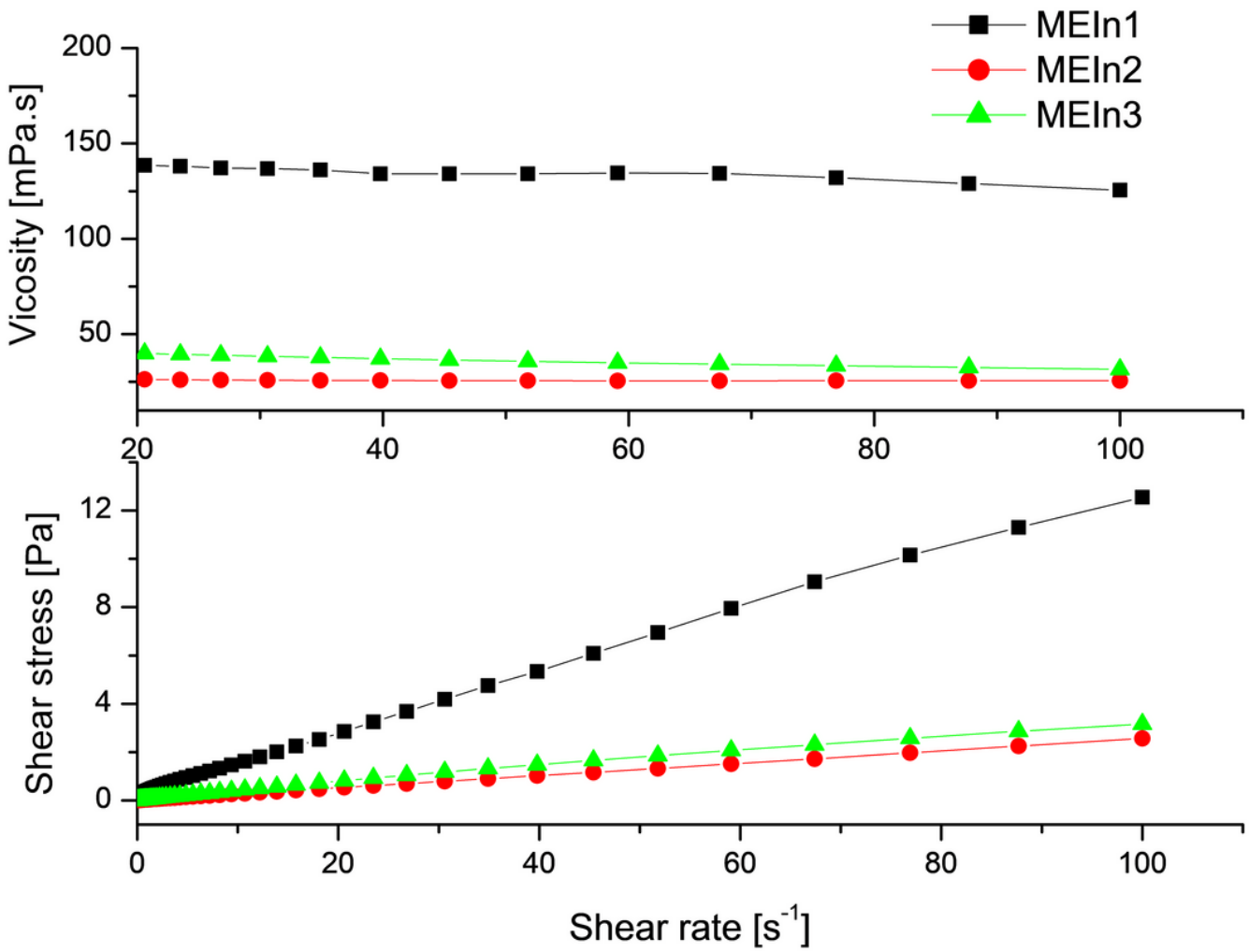

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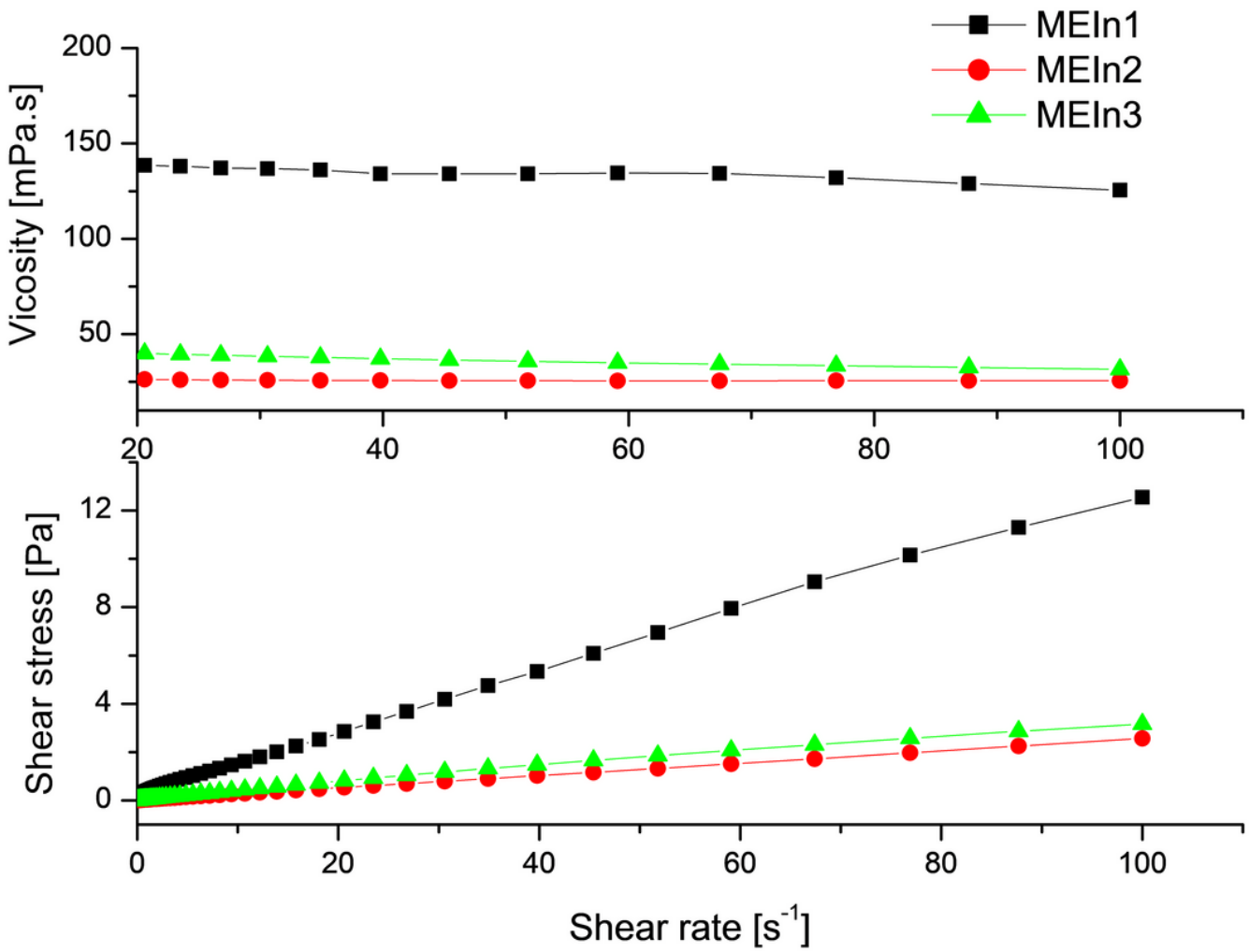

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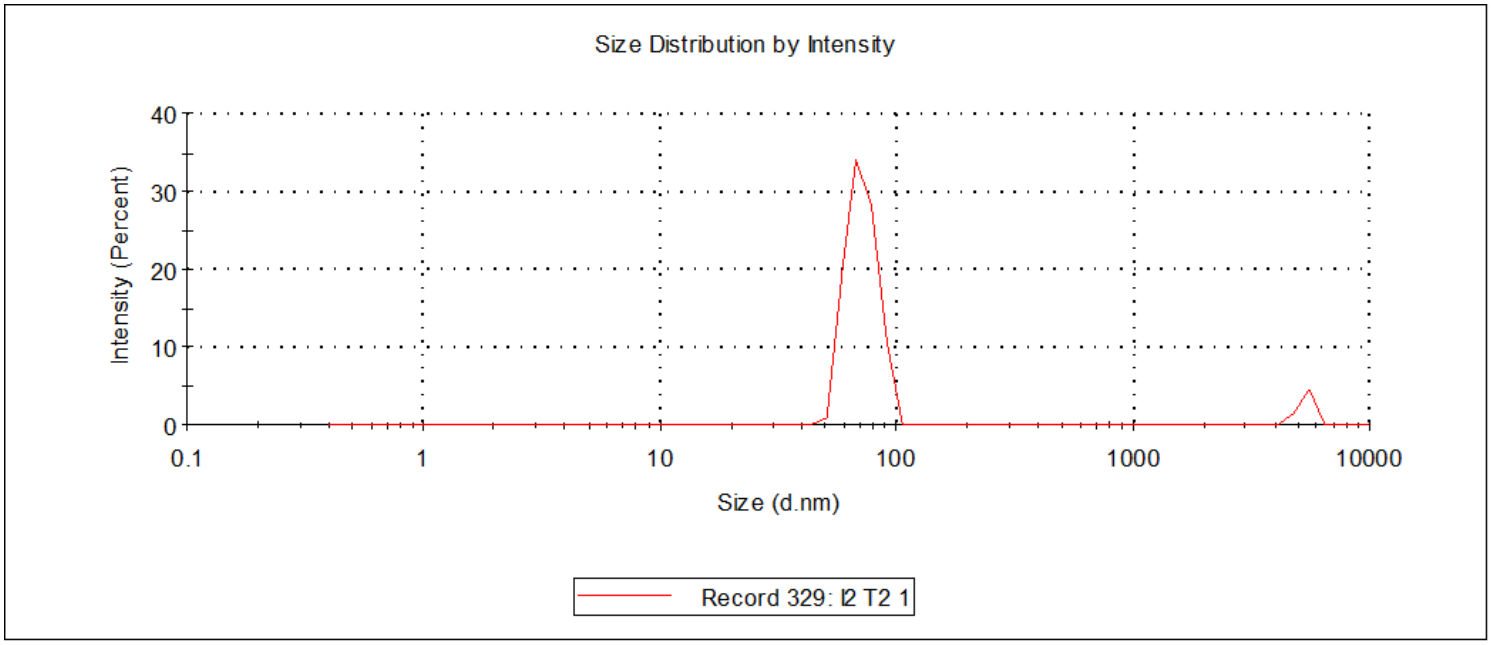
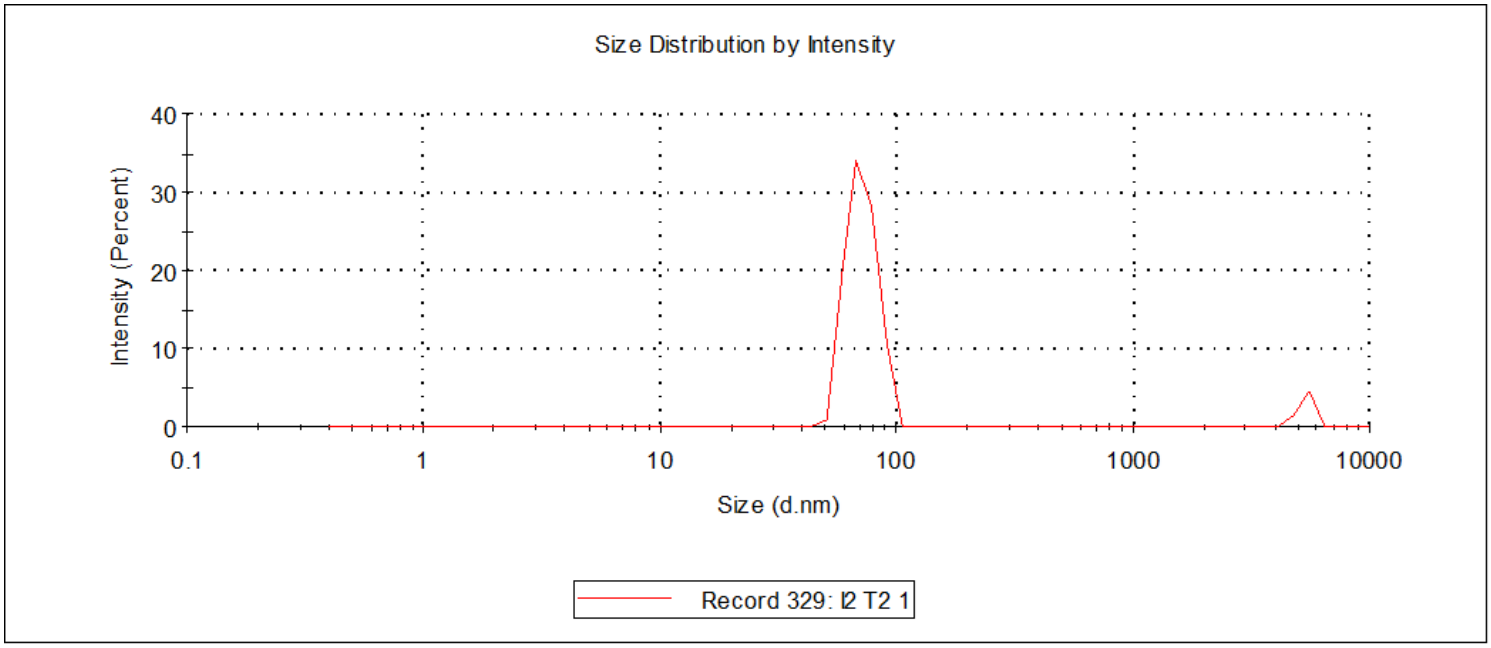


Figure 2

Droplet size distribution of MEIn2 using zeta sizer



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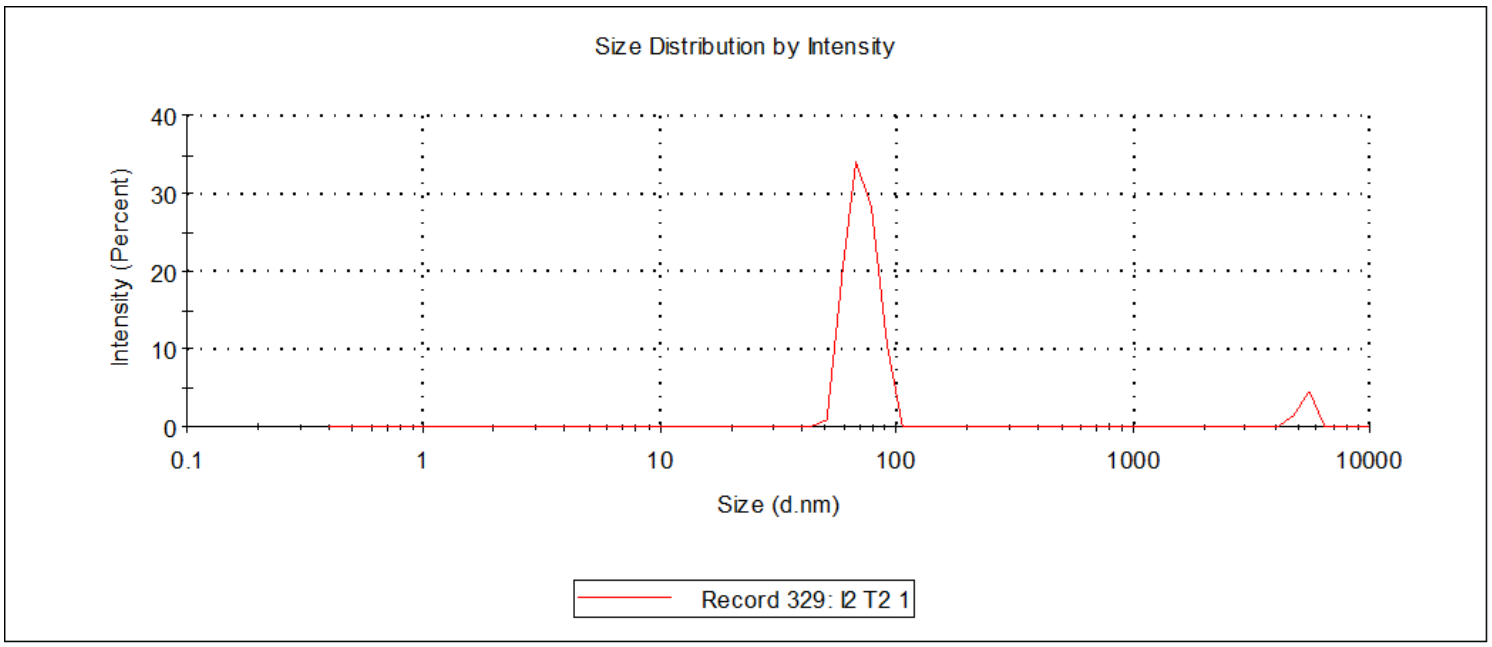

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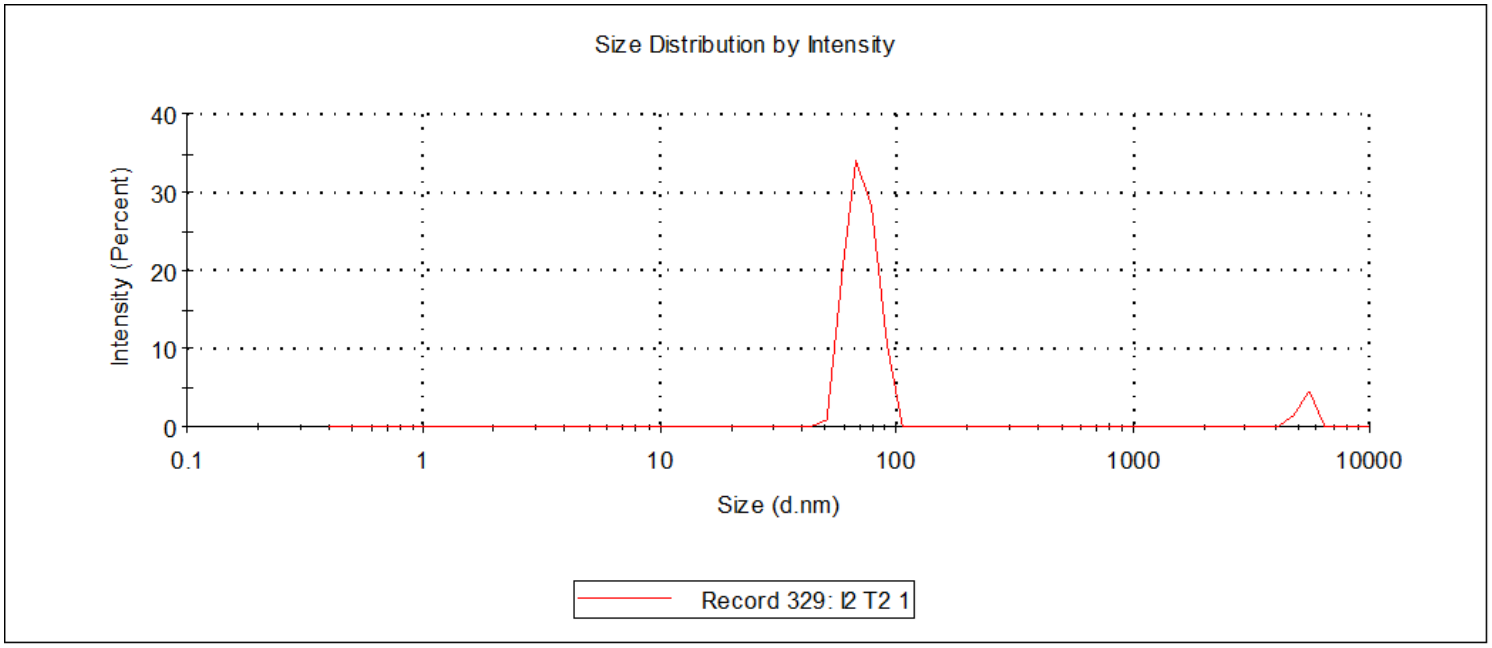

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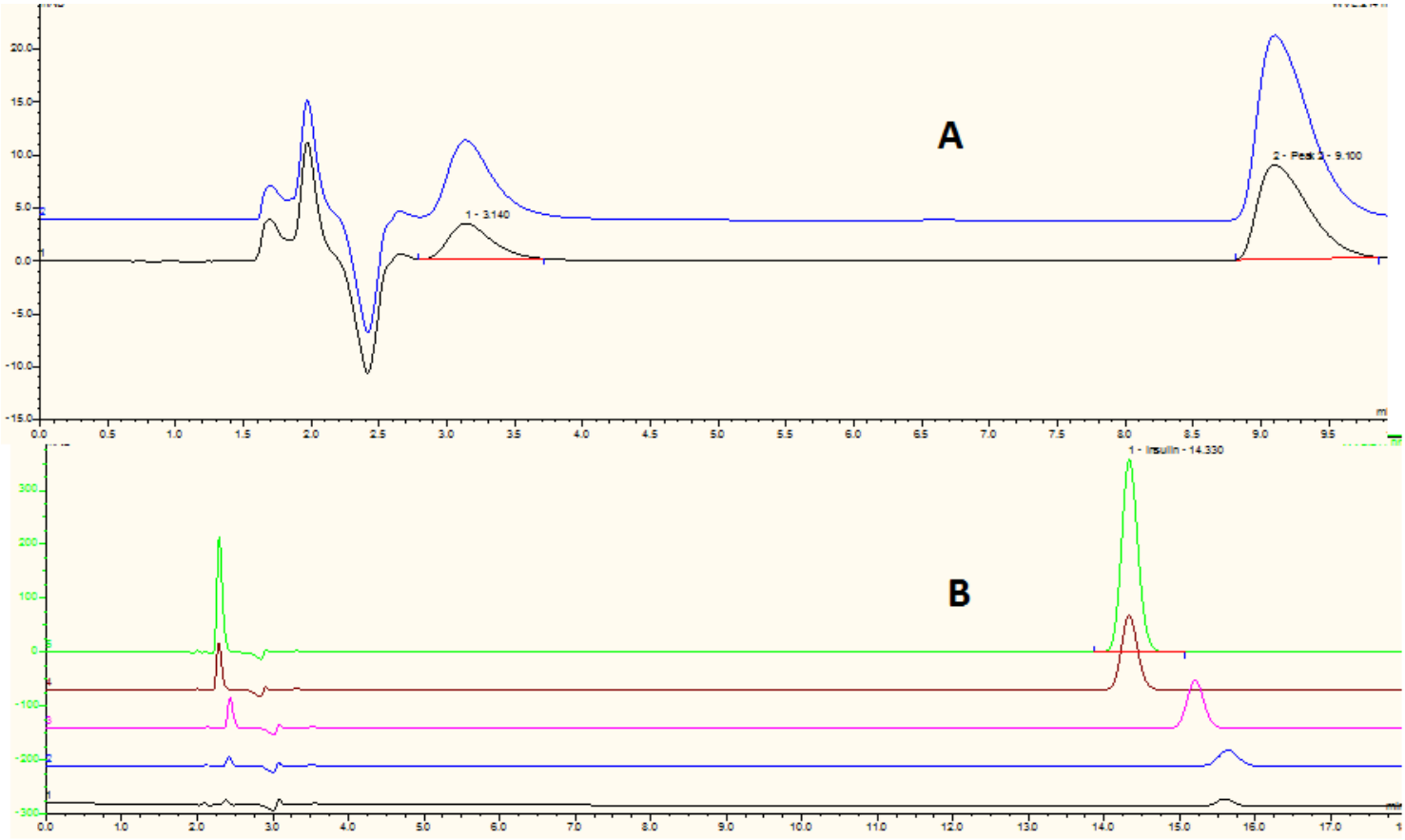

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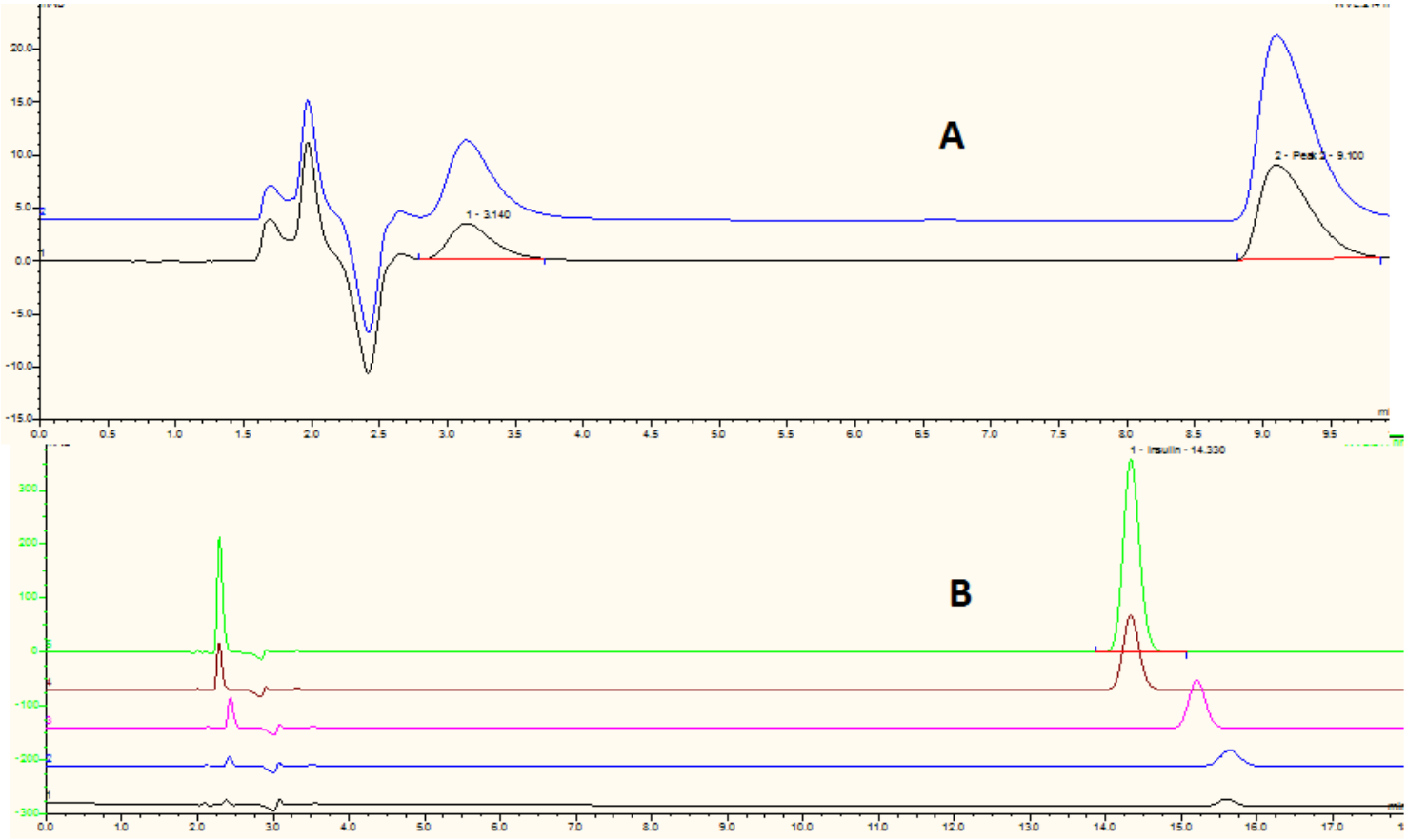
Chromatogram of insulin of A: insulin solution of rapid injection solution; B: prepared insulin solution using 0.01M HCl.



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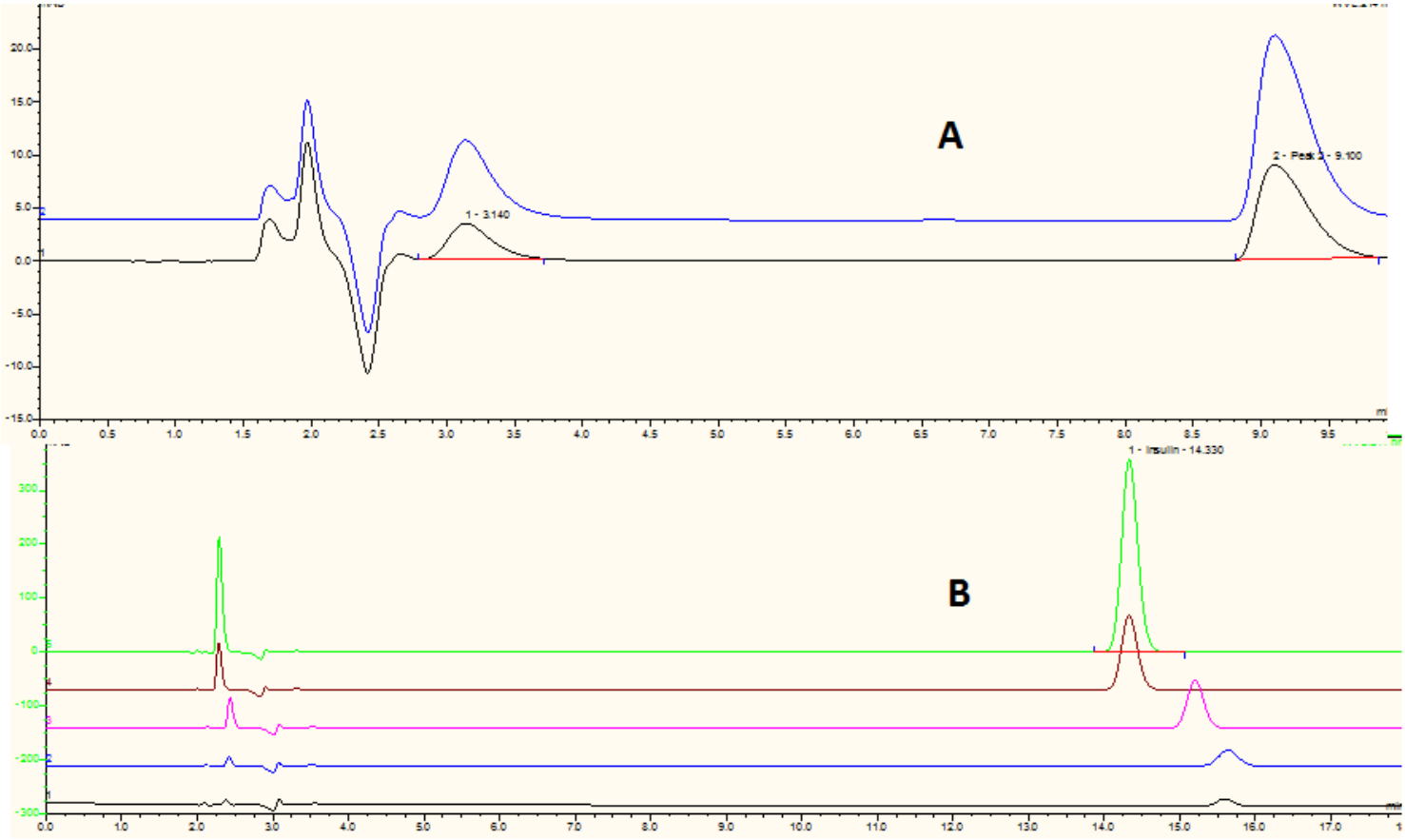

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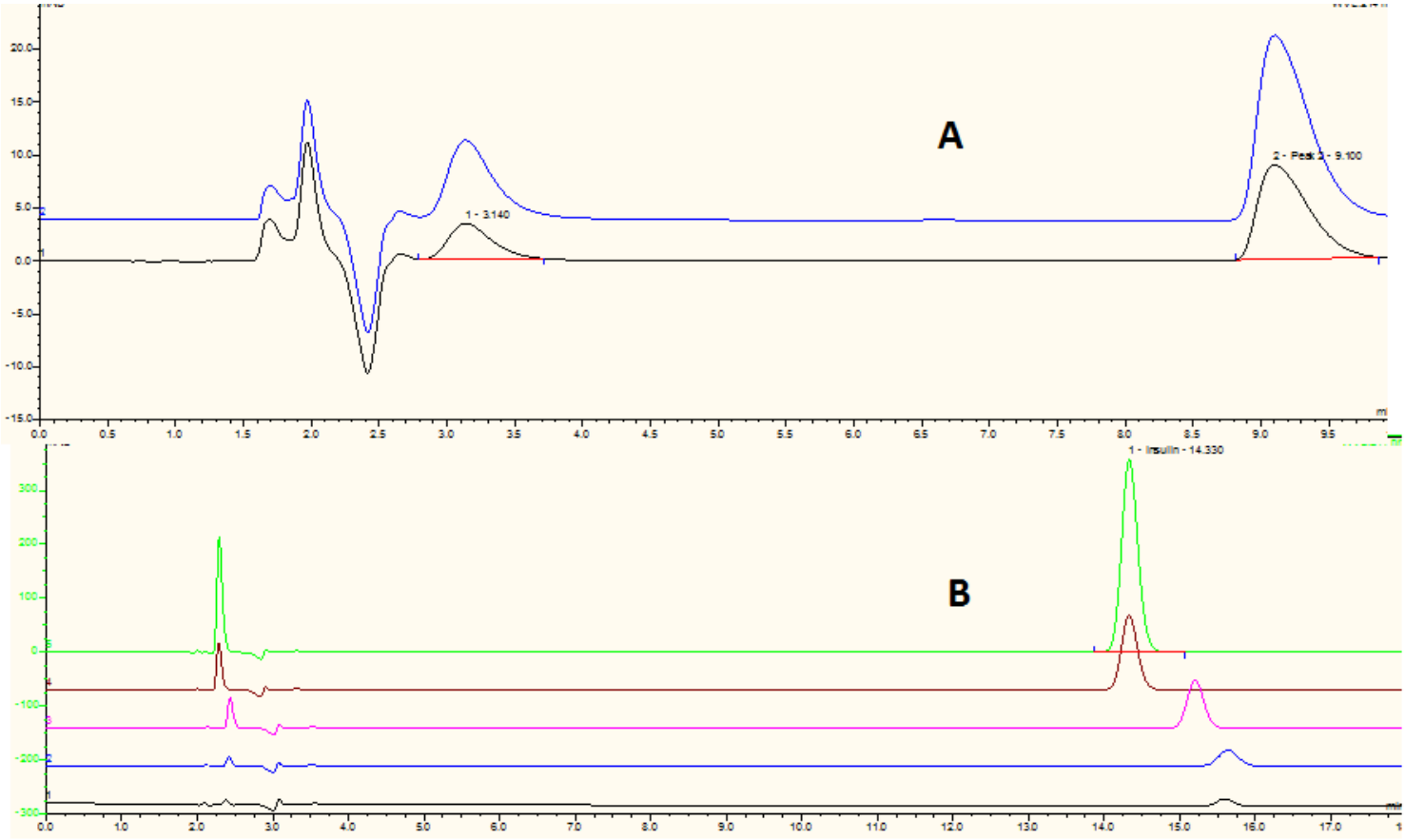

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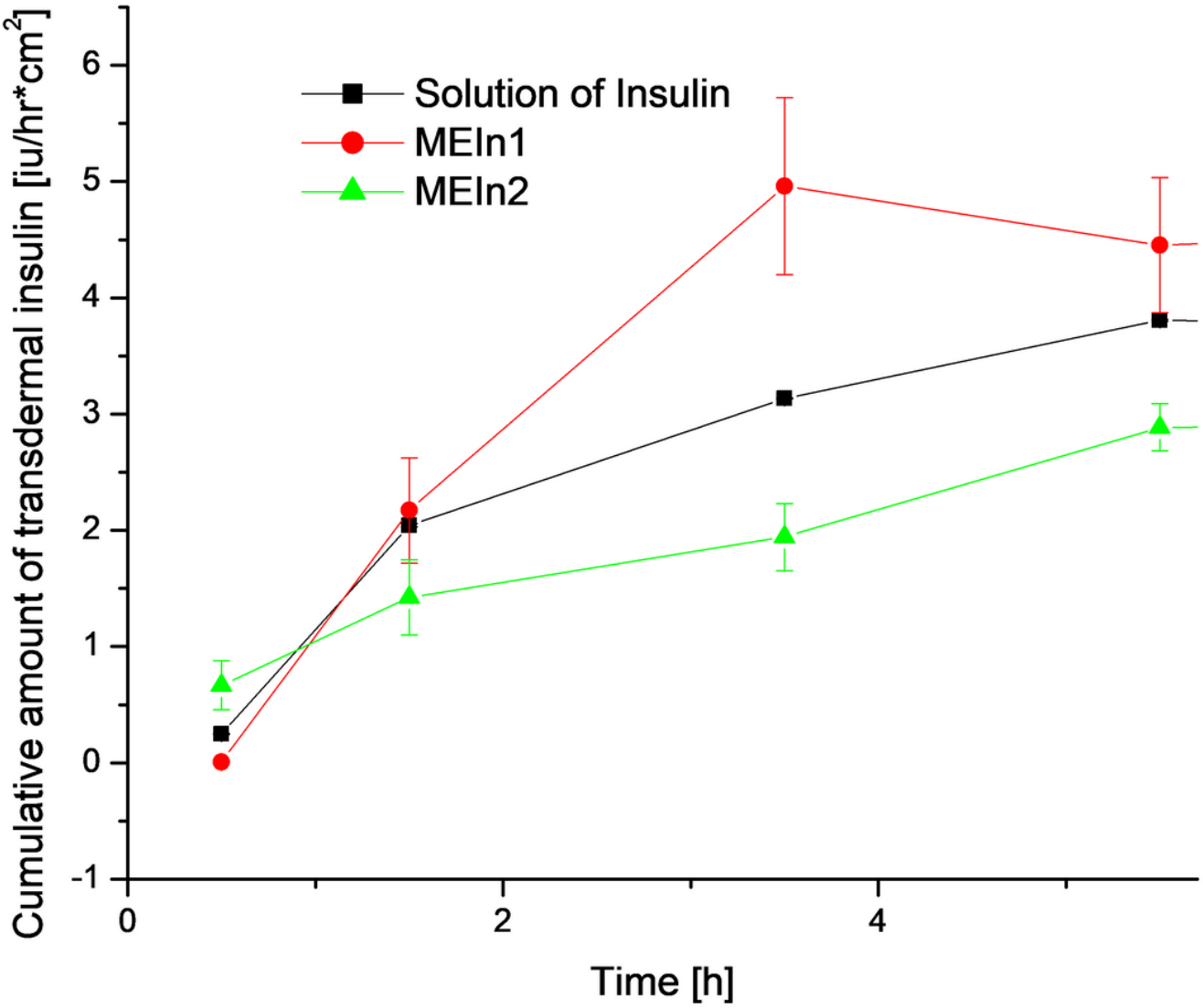

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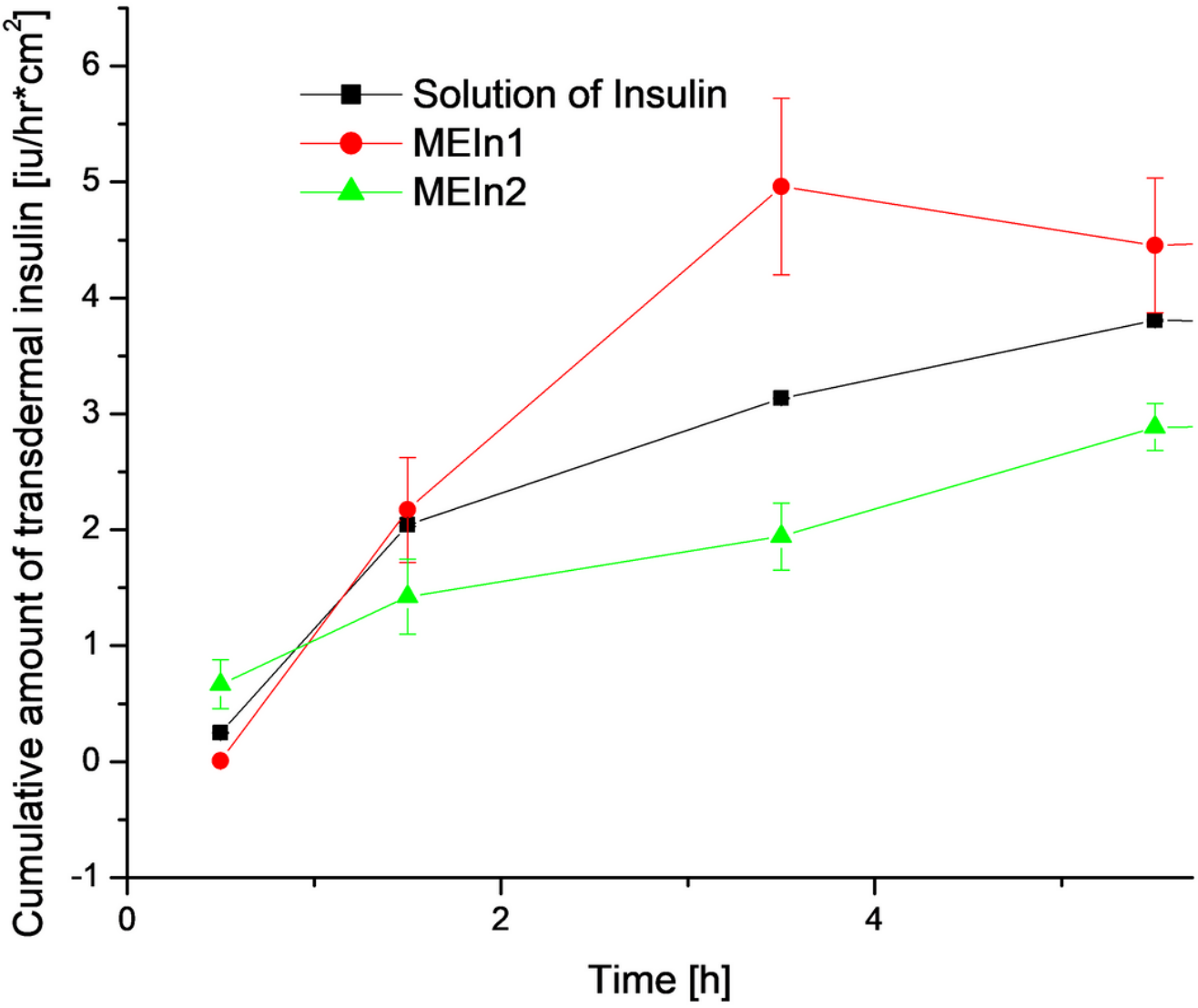

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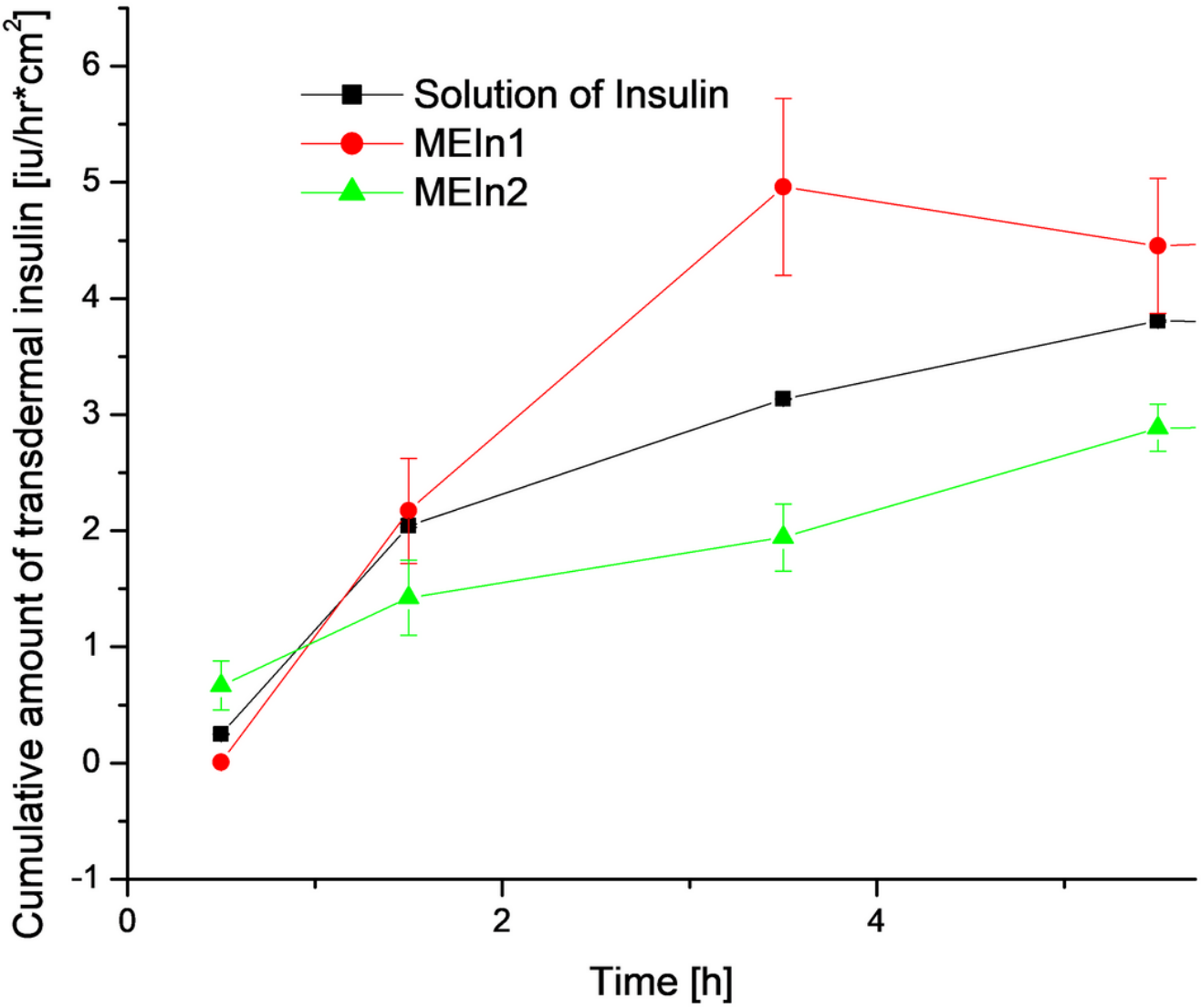

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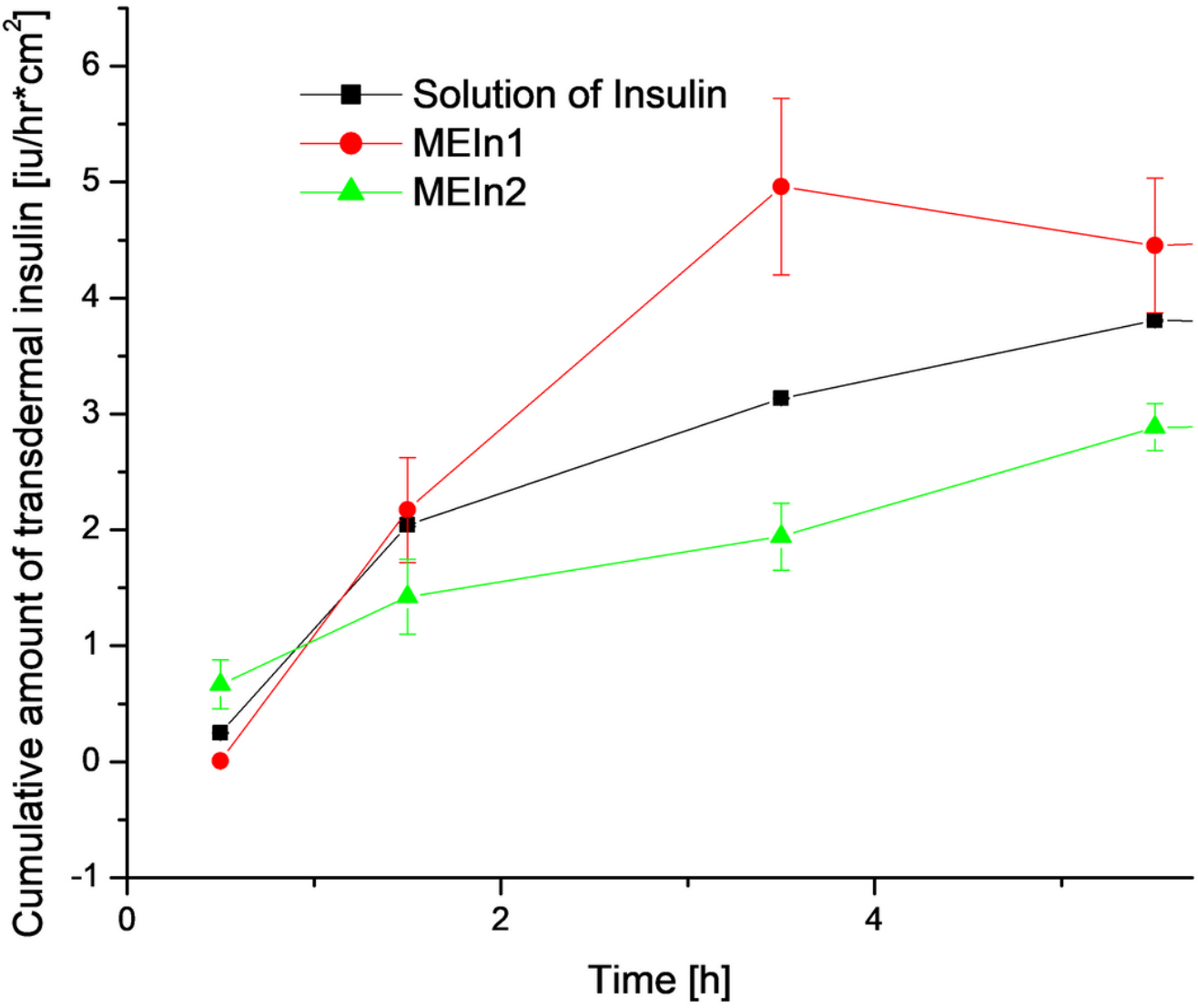

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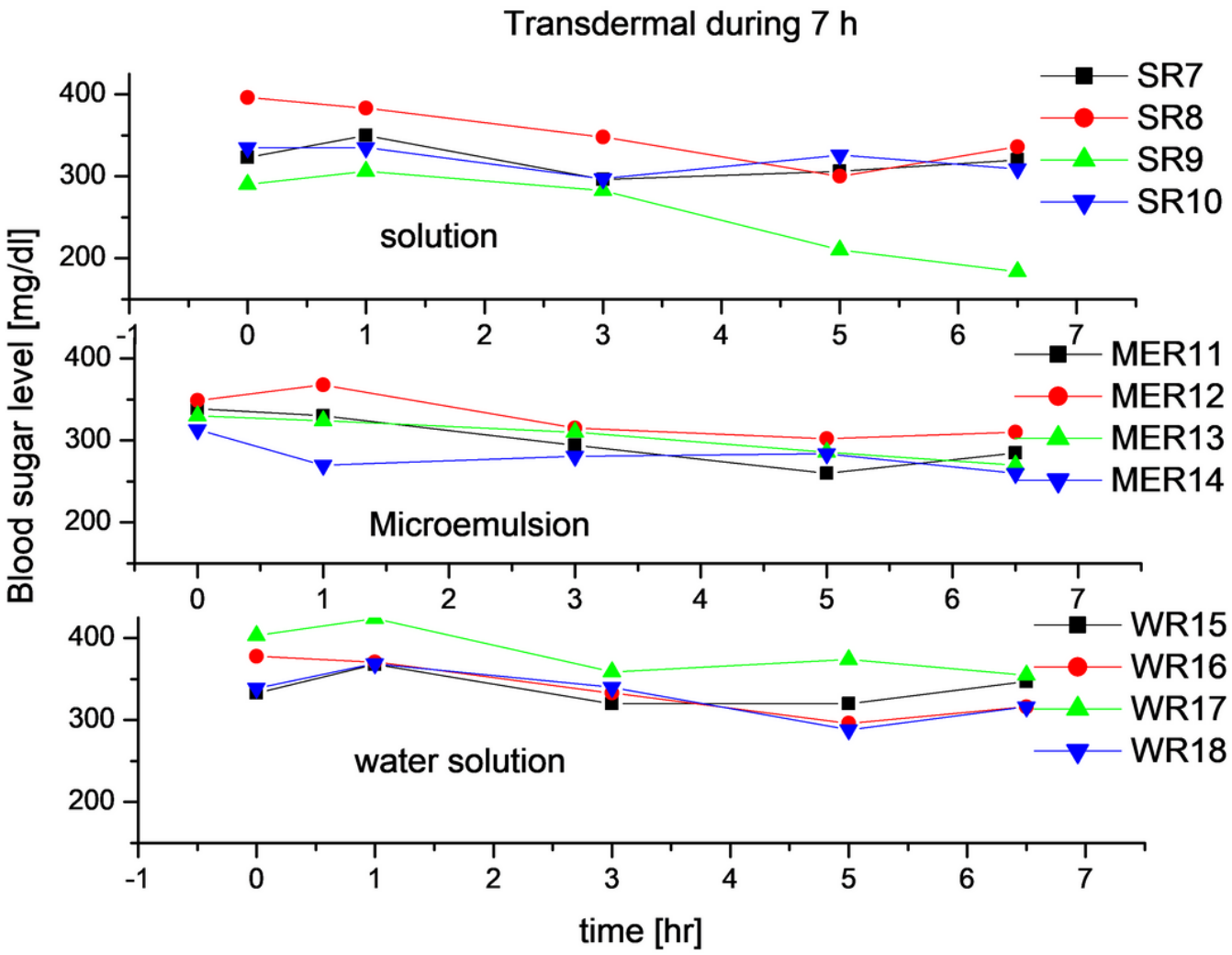
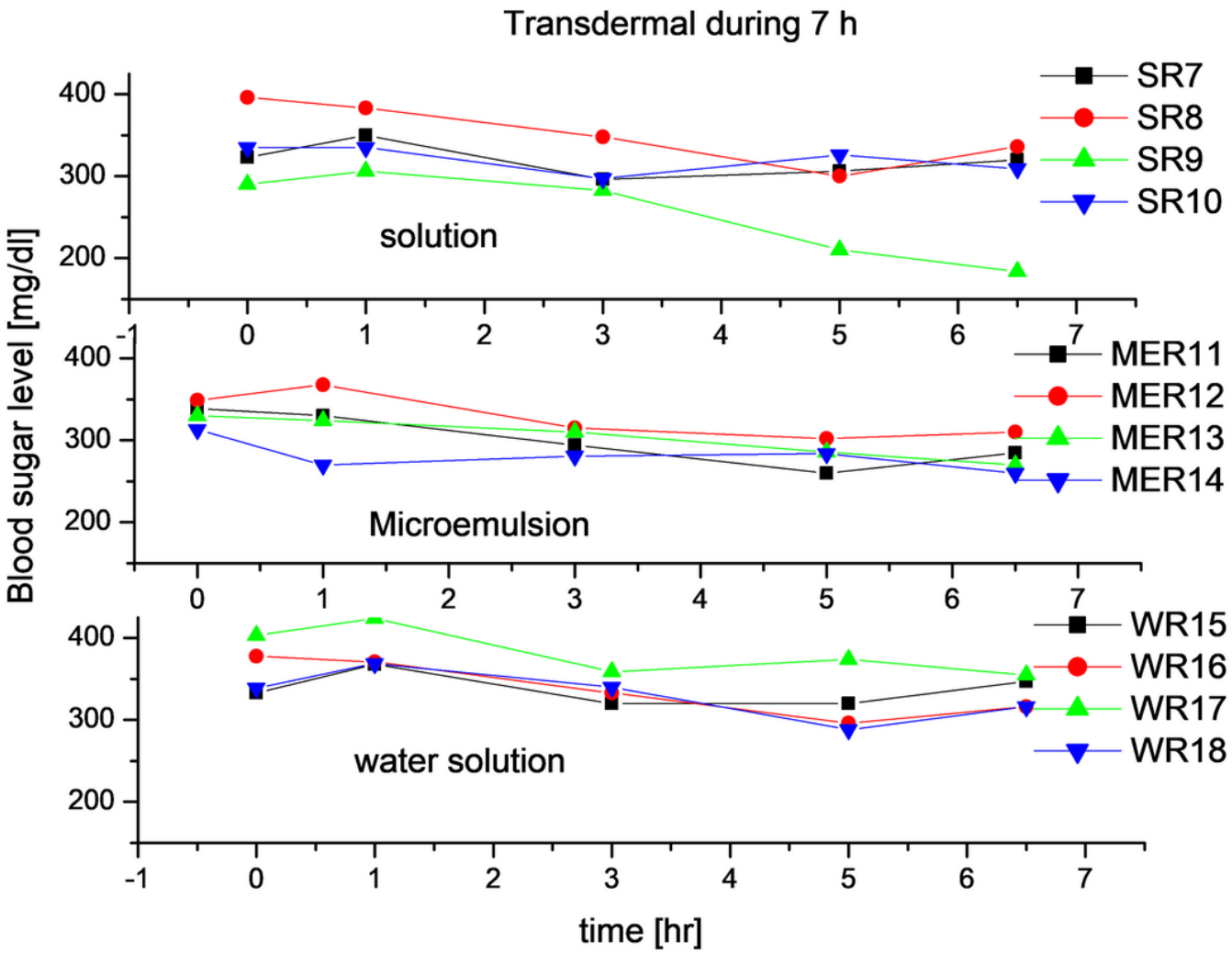


Figure 5

Blood sugar level during 6:30 h in rats (7-18) after transdermal application of insulin using DMSO solution (SR 7-10), MEIn2 (MER 11-14) and water solution of insulin (WR 15-18)



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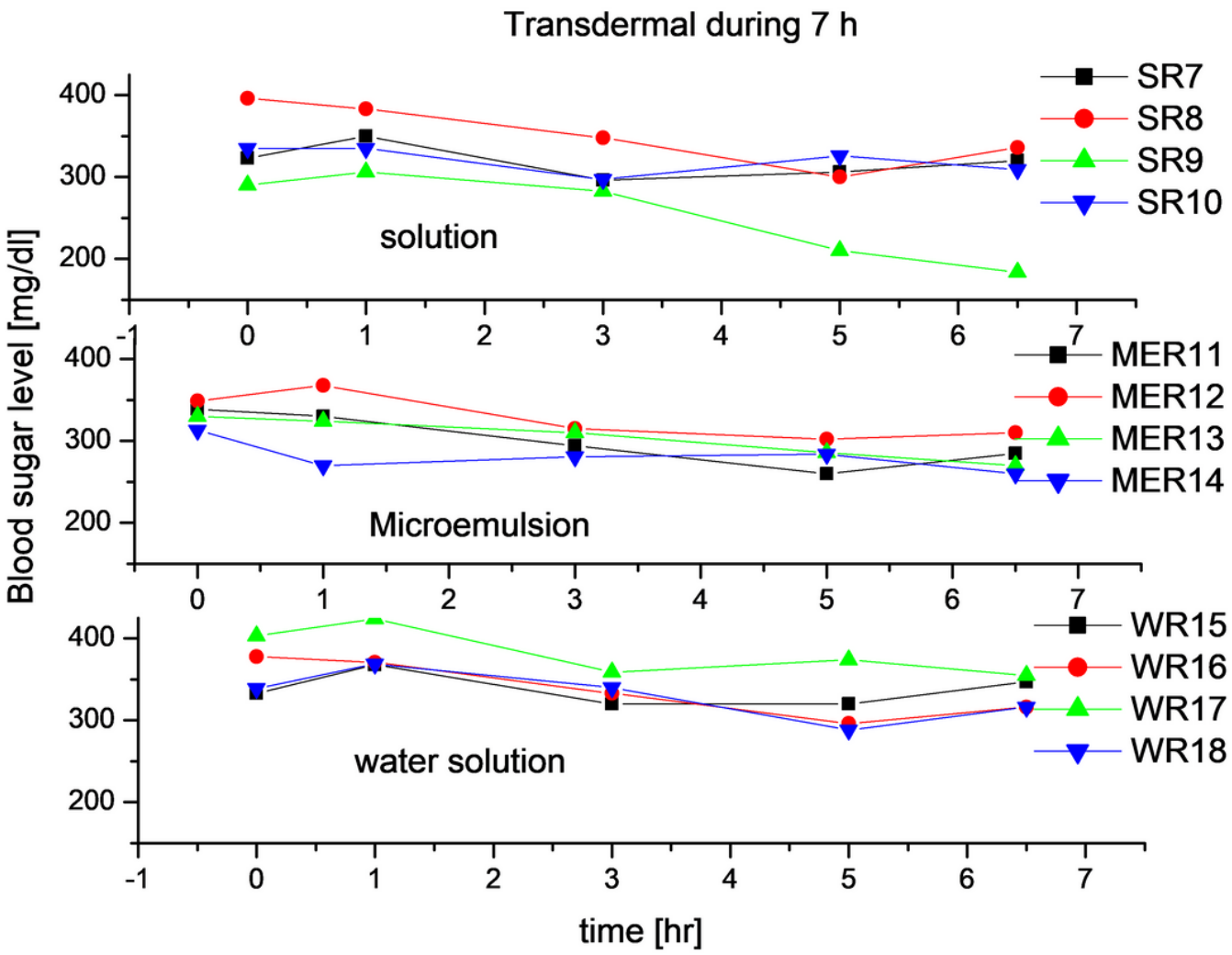

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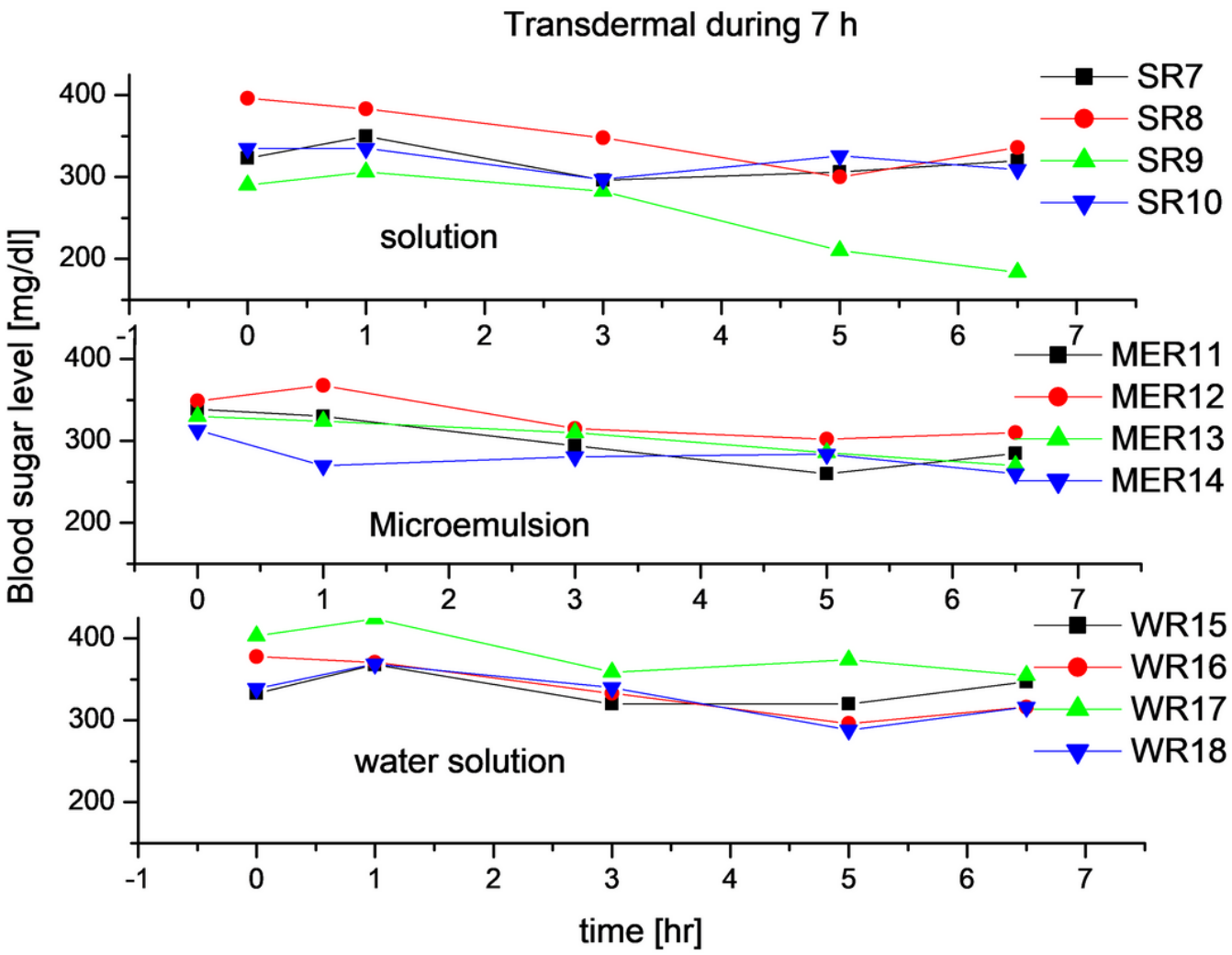

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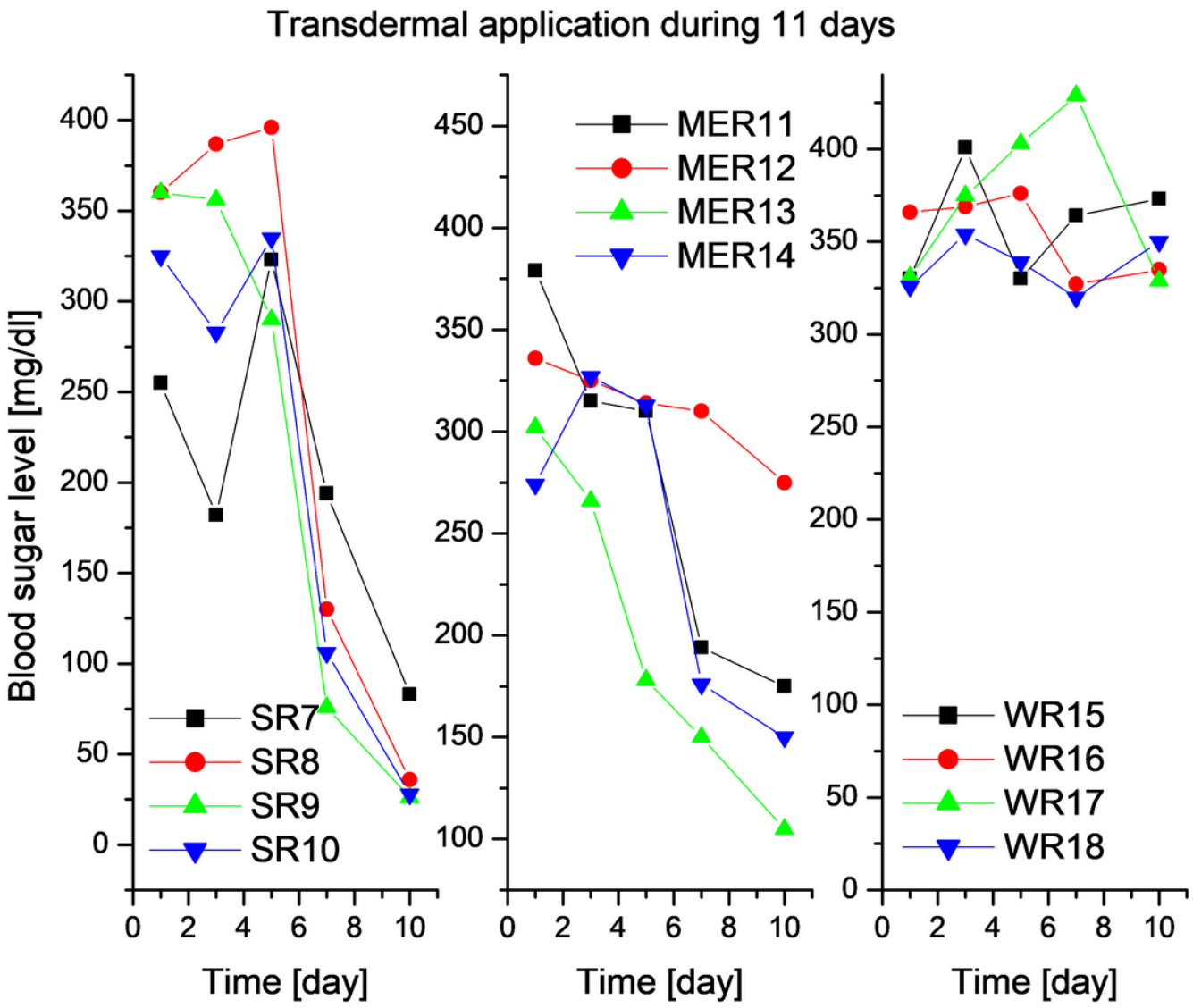

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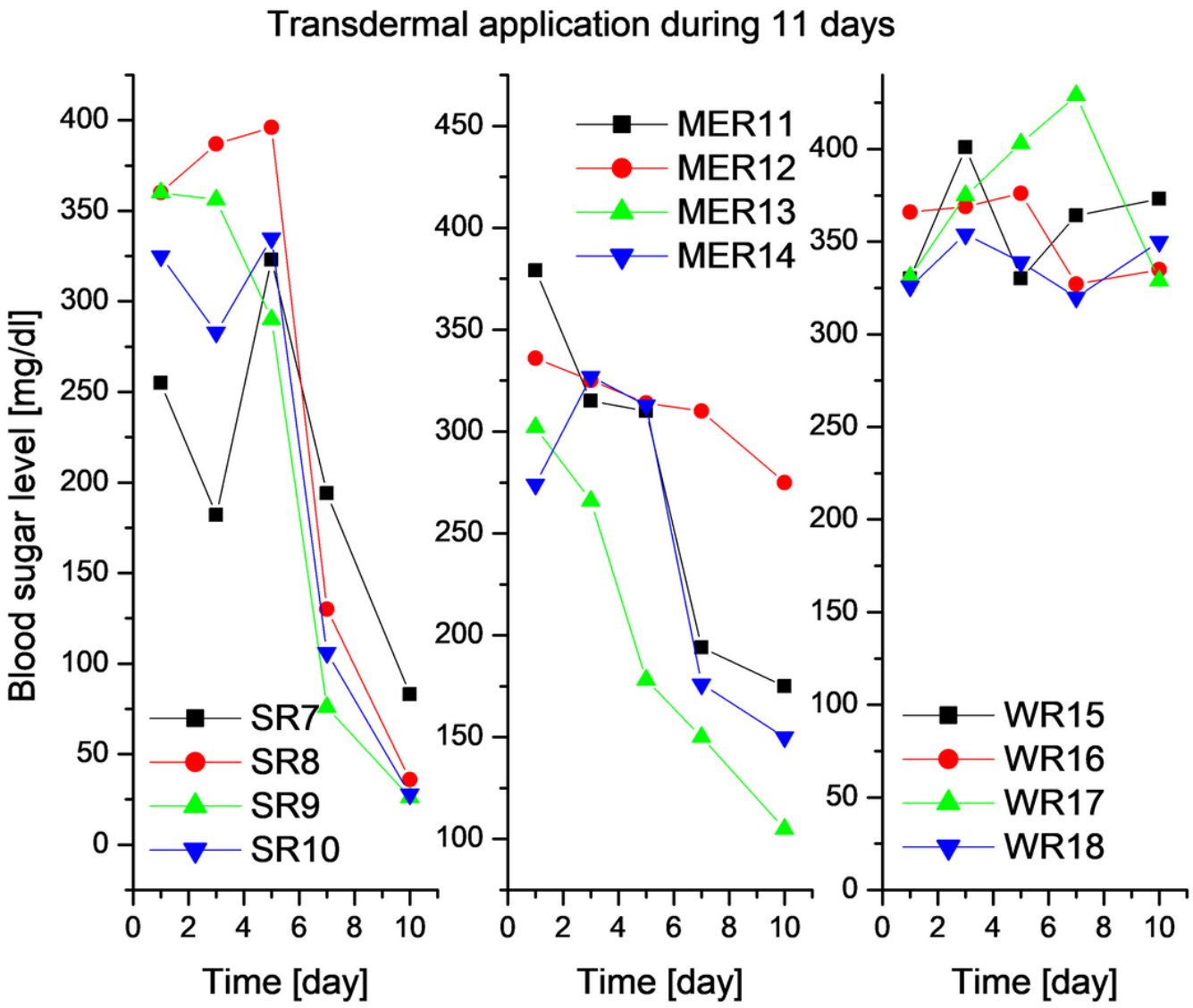

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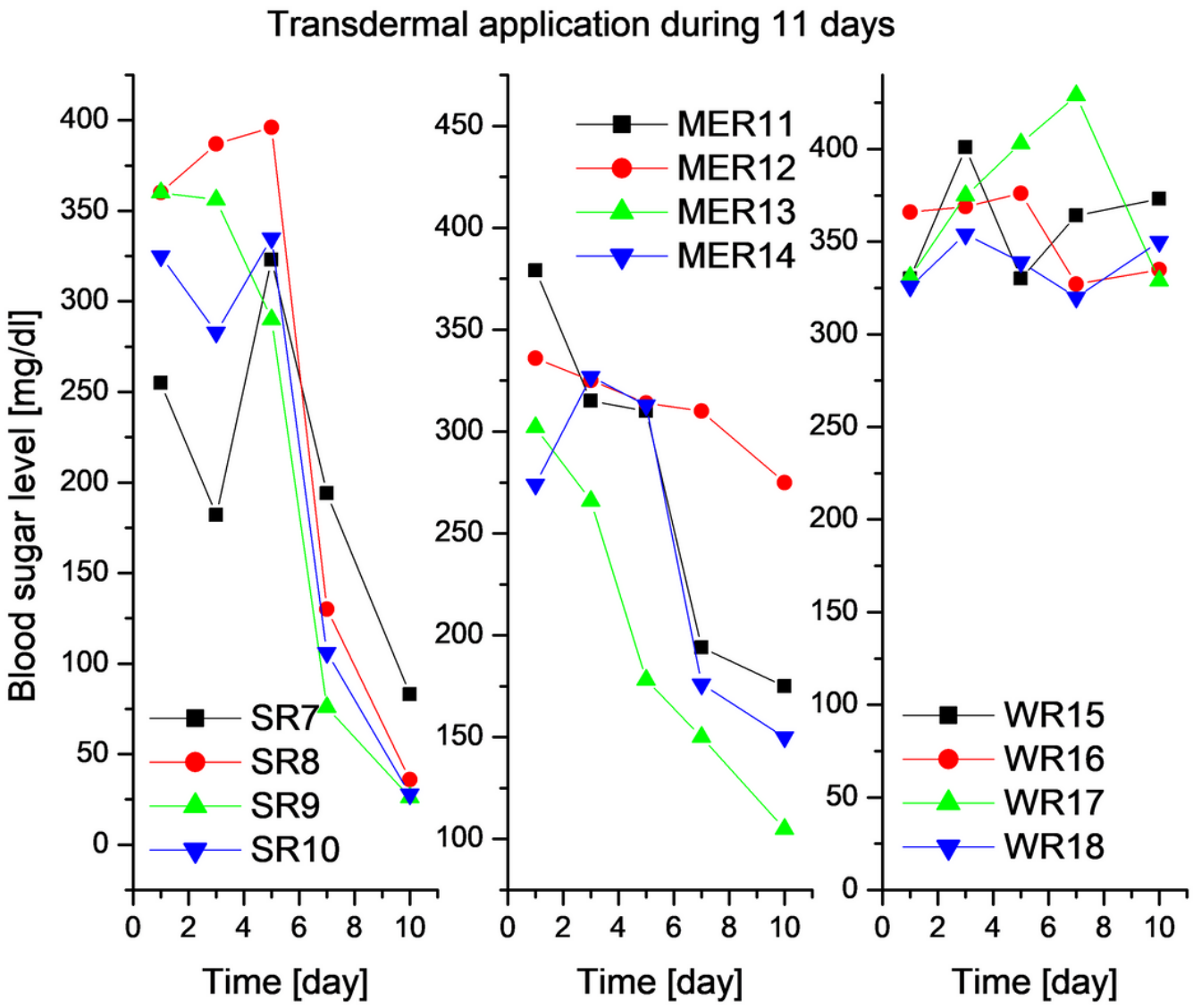

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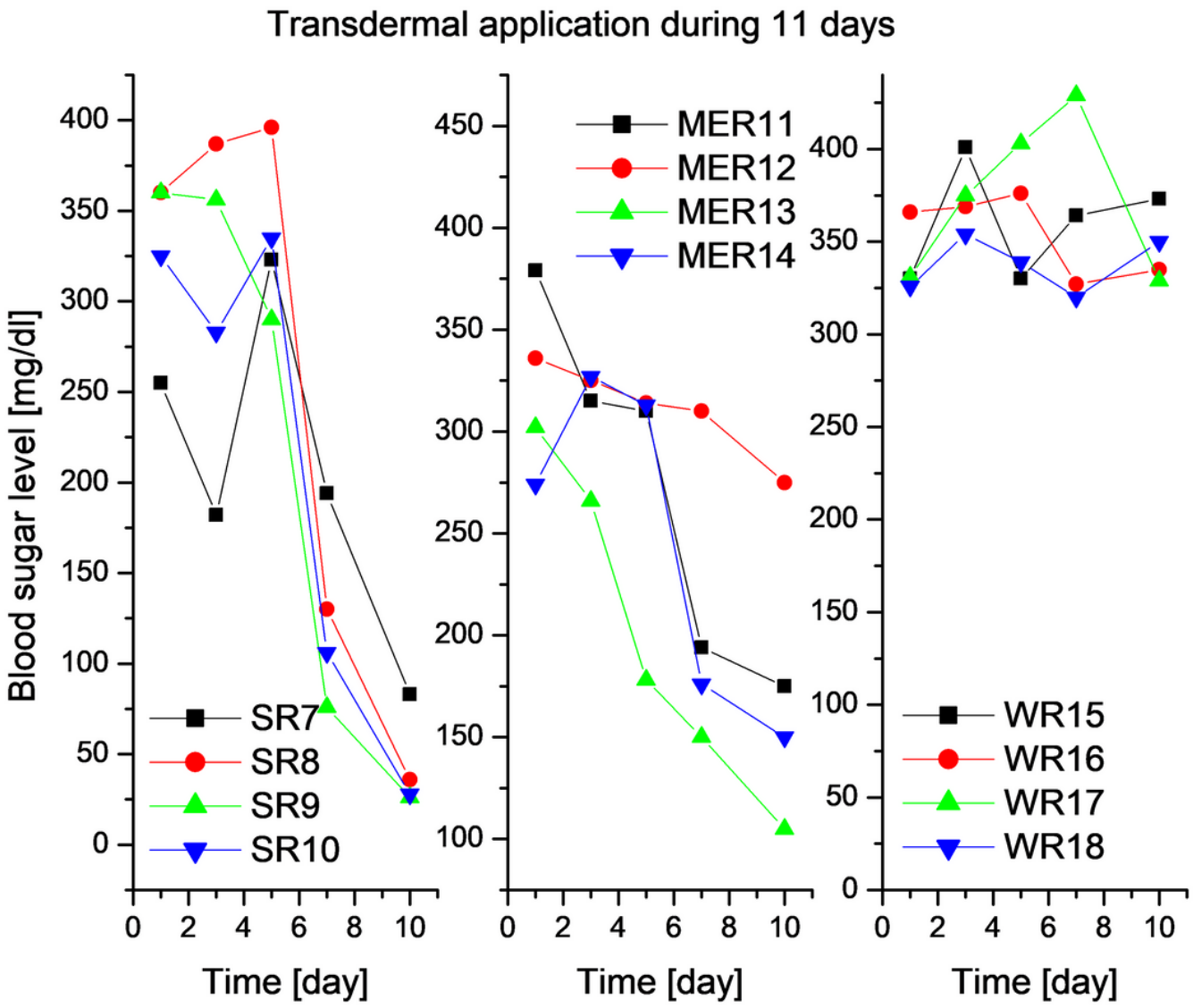

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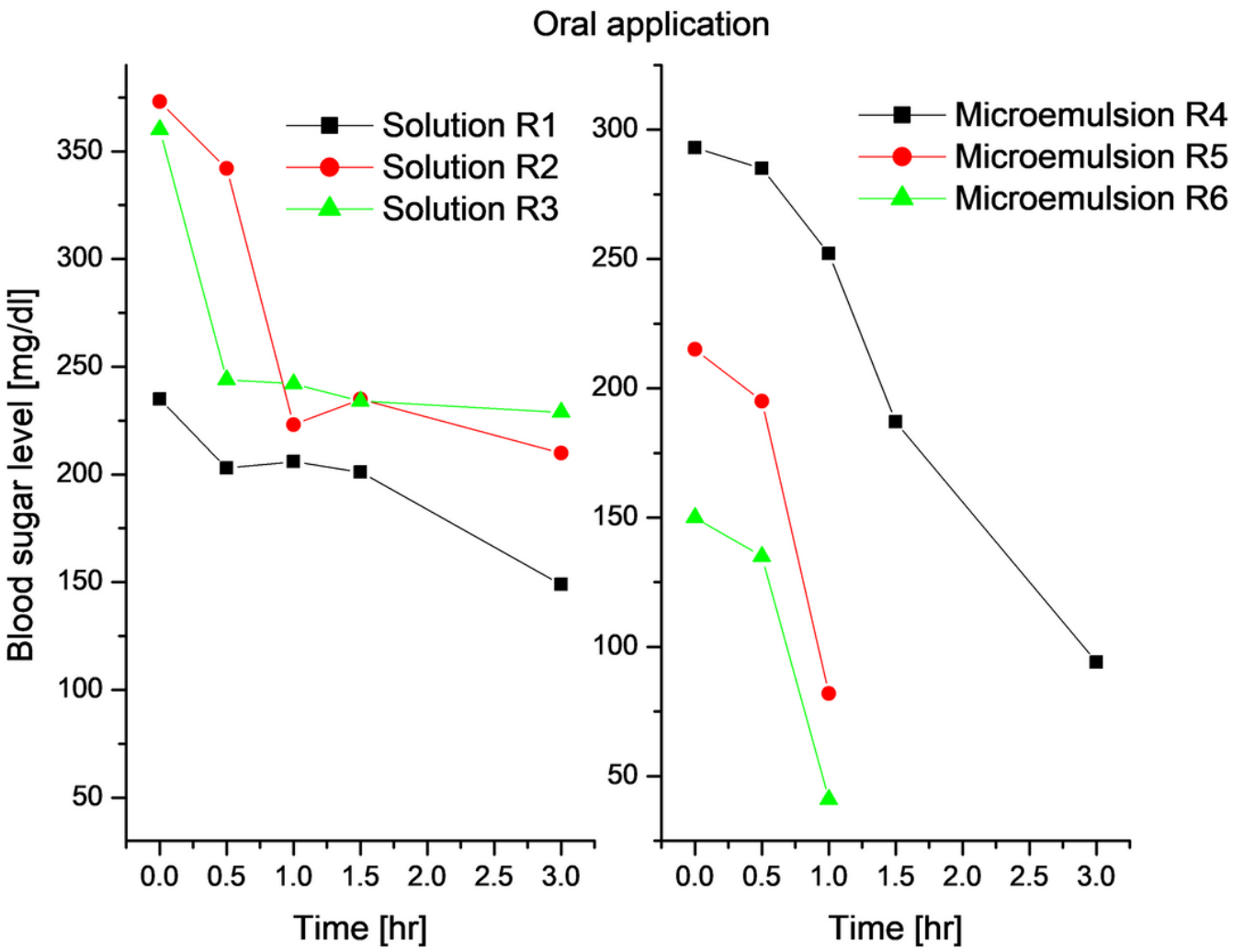

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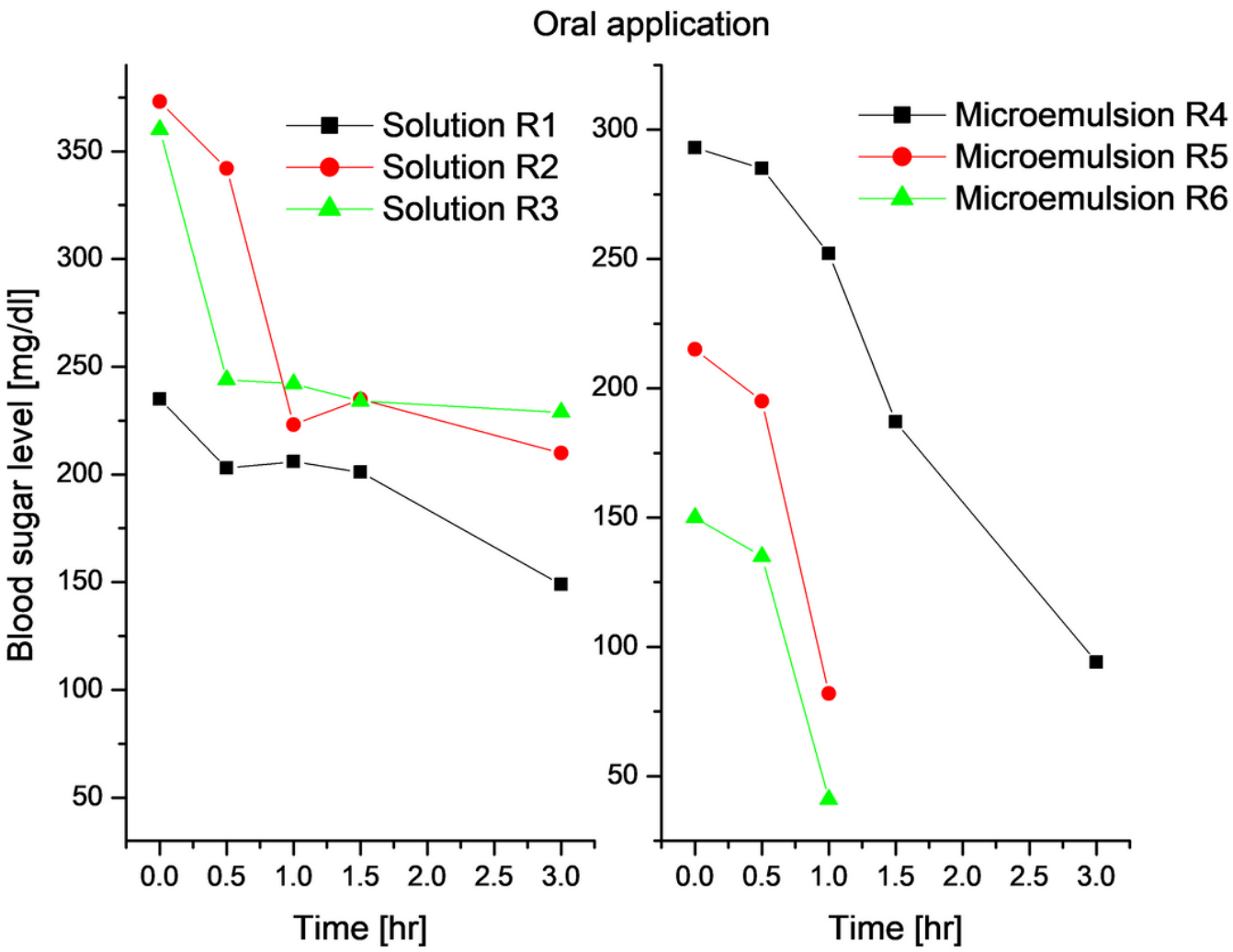

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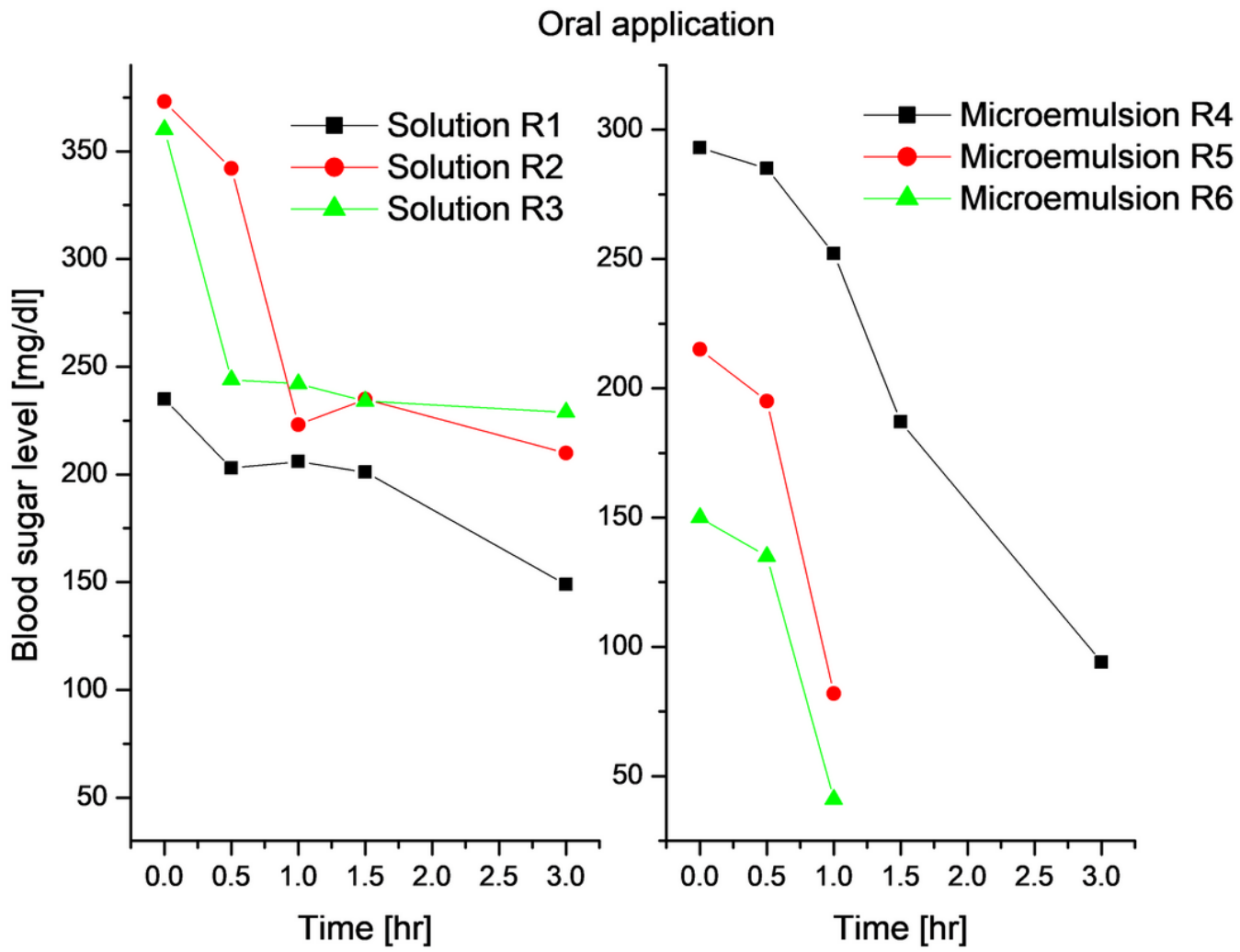

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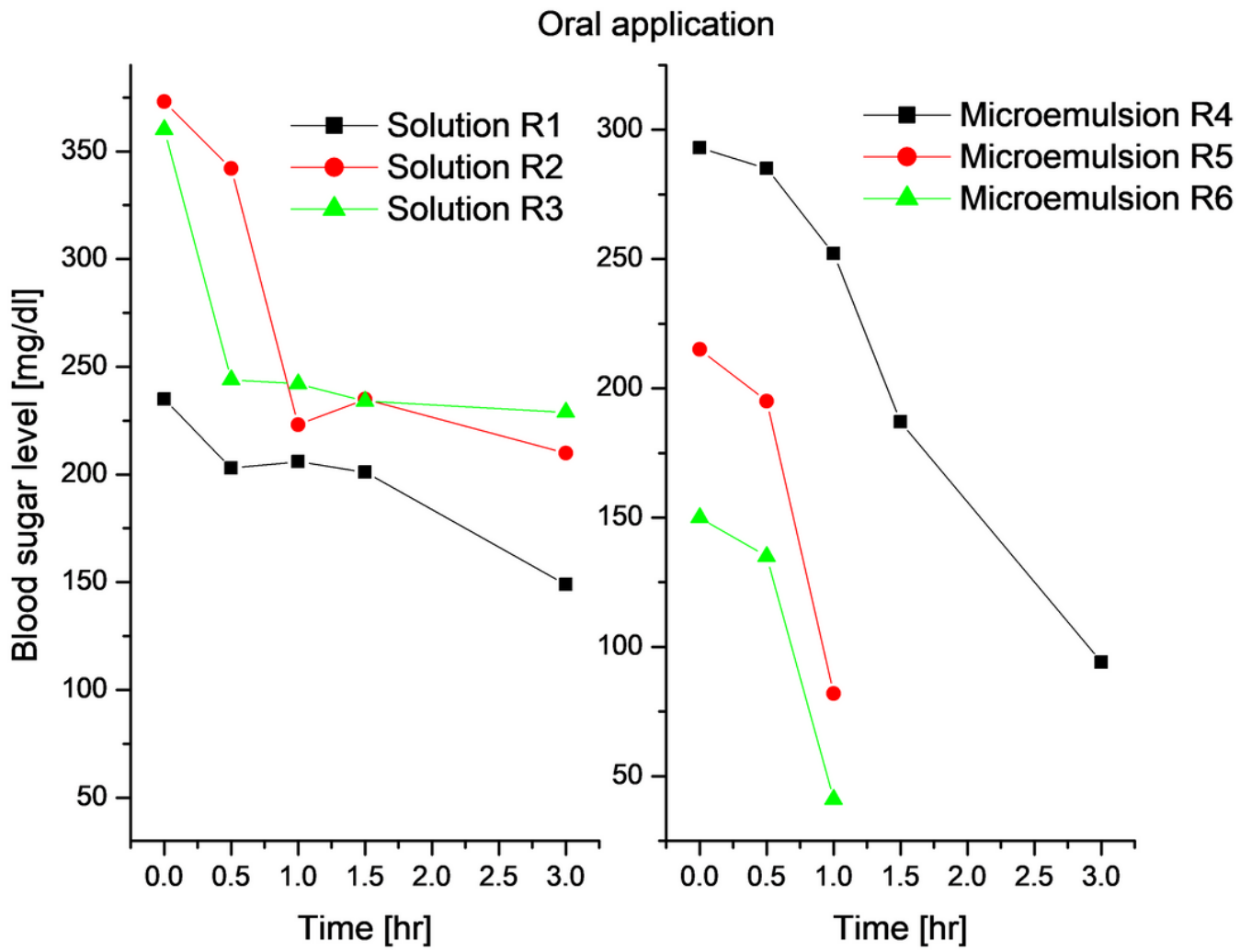

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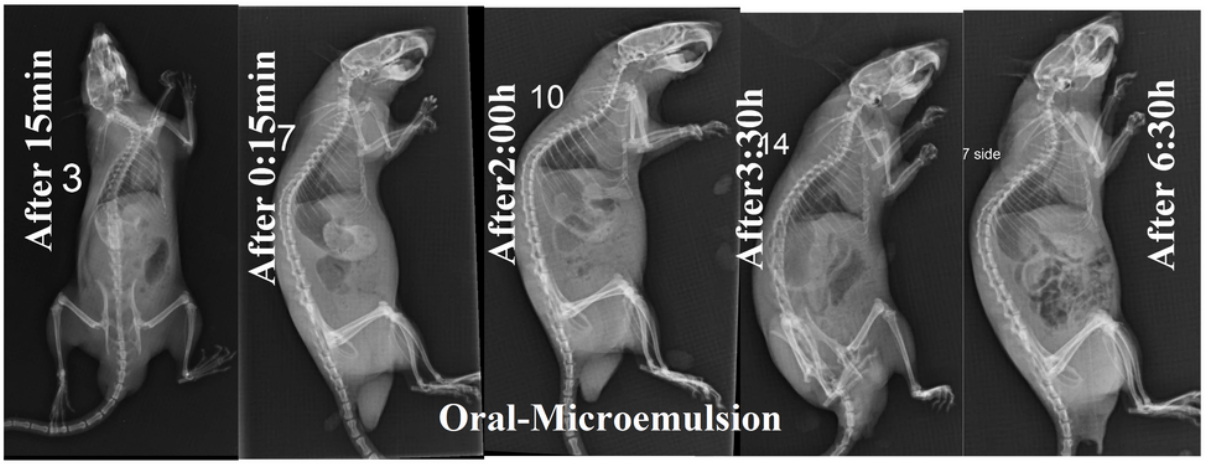

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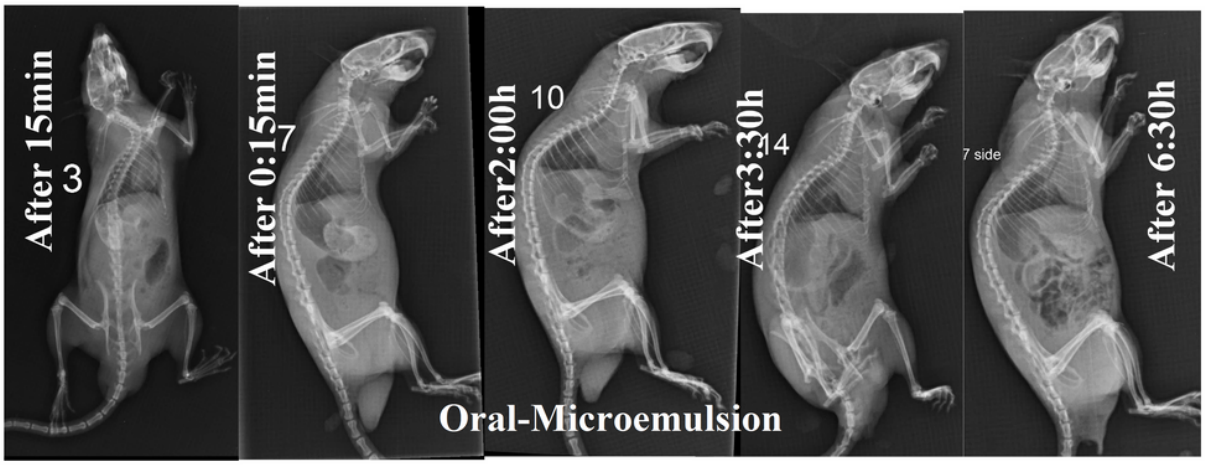

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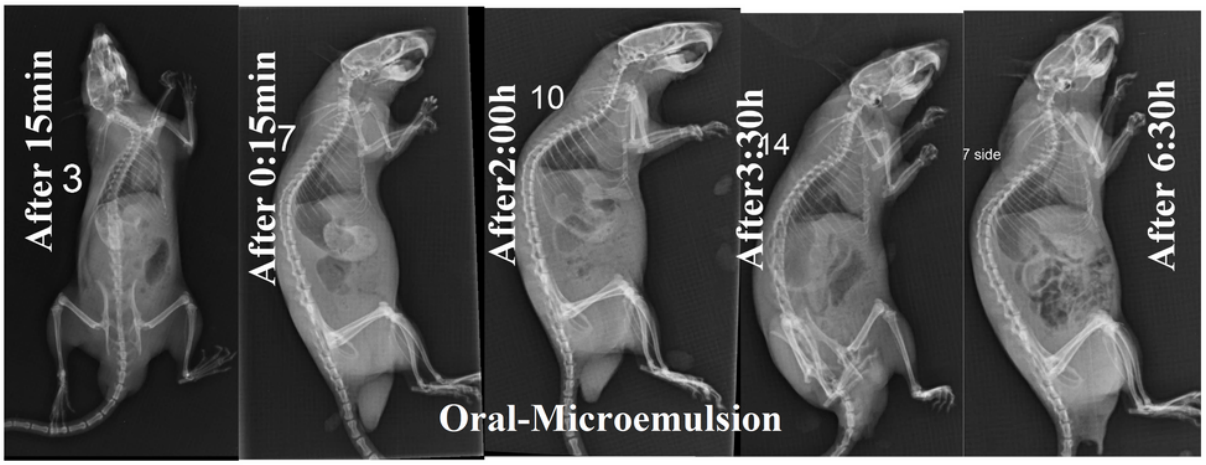

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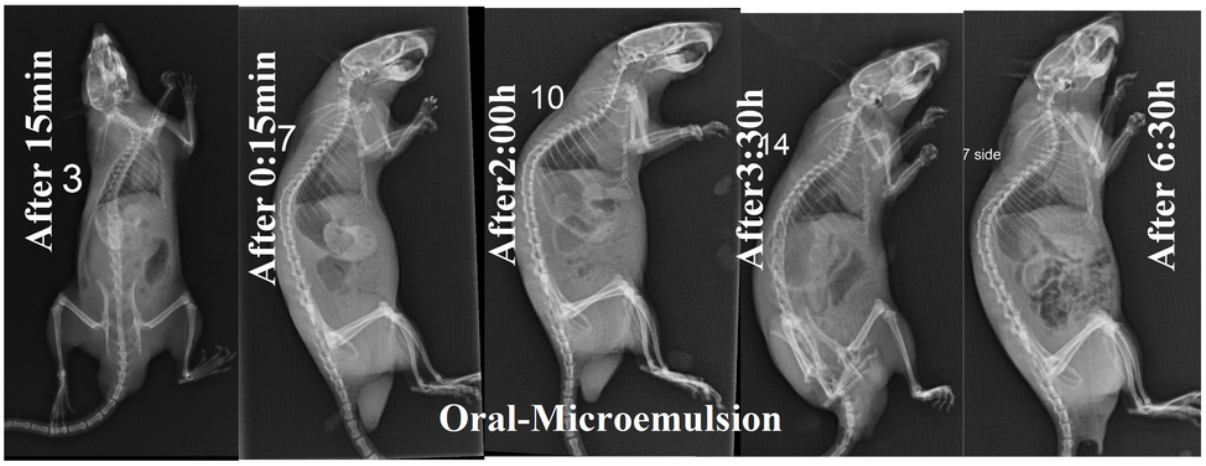

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