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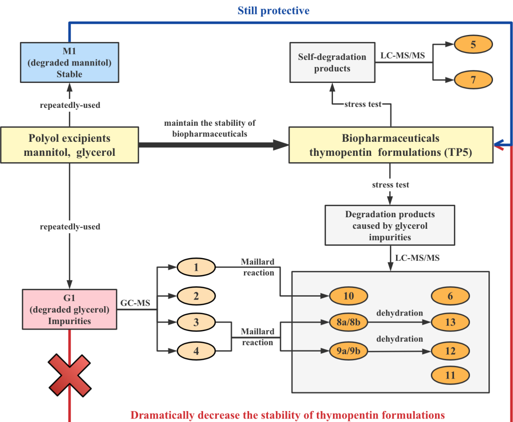


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**Abstract**

Biopharmaceuticals are formulated using a variety of excipients to maintain their storage stability. However, some excipients are prone to degradation during repeated use and/or improper storage, and the impurities generated by their degradation are easily overlooked by end users and are usually not strictly monitored, affecting the stability of biopharmaceuticals. In this study, we evaluated the degradation profile of polyol excipient glycerol during repeated use and improper storage and identified an unprecedented cyclic ketal impurity using gas chromatography with mass spectrometry (GC–MS). The other polyol excipient, mannitol, was much more stable than glycerol. The effects of degraded glycerol and mannitol on the stability of the model biopharmaceutical pentapeptide, thymopentin, were also evaluated. The thymopentin content was only 66.4% in the thymopentin formulations with degraded glycerol, compared to 95.8% in other formulations after the stress test. Most glycerol impurities (i.e., aldehydes and ketones) react with thymopentin, affecting the stability of thymopentin formulations. In conclusion, this work suggests that more attention should be paid to the quality changes of excipients during repeated use and storage. Additional testing of excipient stability under real or accelerated conditions by manufacturers would help avoid unexpected and painful results.

**Keywords:** excipient stability; GC–MS; glycerol; LC–MS/MS; mannitol; thymopentin.

## 1. Introduction

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33 Biopharmaceuticals generally have complex and fragile structures compared with traditional
34 small-molecule drugs [1,2]. Optimization of formulations, that is, the addition of excipients, is a
35 common method for maintaining their storage stability [3]. Carbohydrates, polyols, amino acids,
36 buffers, and surfactants are the major excipients used to stabilize proteins in biopharmaceutical
37 formulations [4]. These excipients significantly alter the conformational and colloidal stability of
38 proteins and reduce their chemical degradation through interactions with drugs [5−7]. For example,
39 carbohydrates and polyols stabilize proteins through various alternative mechanisms to improve their
40 colloidal stability [8,9].

41 Biocompatible pharmaceutical excipients are generally chemically inert. In practice, they may
42 undergo degradation during production, repeated use, or storage. Owing to the different excipient
43 manufacturing processes of various manufacturers, the quality and stability of the preparation are
44 affected by the purity and contamination level of the excipients [10]. Trace metals, for example, are
45 inherent impurities in common excipient production processes and are ubiquitous in almost all
46 excipients [11,12]. In addition, carbohydrate and polyol excipients frequently contain reducing
47 impurities during the production process [13,14]. Nevertheless, impurities in the production process
48 of excipients can often be traced back to their sources, which can be controlled by optimizing
49 production conditions and purification, and many related studies have been conducted to ensure
50 product quality.

51 Other impurities in pharmaceutical excipients, such as those produced during storage and use,
52 have not attracted enough attention. For pure excipients that meet the release standard, various
53 degradations may occur during storage and use, leading to potential reactions with drugs [11]. For
54 example, since the excipients in chemotherapy are a large part of the dose, accounting for up to 99%
55 of the total formulation mass [15], there is a significant amount of literature about the effect of
56 excipient quality on chemotherapeutic drugs [16−18]. In contrast, owing to the small number of
57 excipients used for biopharmaceutical preparations, a bottle of excipients will be stored and used for a
58 long time. During this process, they come into contact with oxygen and moisture, leading to the
59 generation of impurities that are easily overlooked. A vast majority of the existing studies have focused
60 on the effect of polysorbates on the physicochemical stability of biopharmaceuticals such as
61 monoclonal antibodies. Donbrow et al. [19] and Kerwin [20] reported that polysorbates may undergo
62 autooxidation, hydrolysis of the fatty acid ester bond, and cleavage of the ethylene oxide subunits
63 during storage and use. Polysorbate autoxidation generates hydrogen peroxide, leading to oxidation of
64 the protein during preparation [21,22]. Its hydrolysis produces free fatty acids, and further studies have
65 revealed that polysorbate-containing biopharmaceutical formulations produce protein particles that
66 contain fatty acids during long-term storage [23,24]. These studies suggest that oxygen-free and low-
67 temperature conditions should be maintained during storage and utilization of polysorbate excipients

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68 [25]. In fact, because of the sensitive physical and chemical stability of biopharmaceuticals, even if
69 the content of excipients in the preparation is small, minor changes in the properties of excipients
70 during storage and use may lead to significant changes in the stability of biopharmaceuticals. Therefore,
71 further studies on the impact of changes in the excipients for biopharmaceuticals during storage and
72 use are needed to increase the industry's awareness of excipient degradation.

73 Glycerol is a liquid polyol with a wide range of applications in the pharmaceutical industry [26].
74 It can be used not only as an oral or intravenous drug, but also as an excipient in pharmaceutical
75 preparations, such as insulin, thymopentin, and freeze-dried biological drugs [27,28]. There are many
76 reports on the oxidation of glycerol [29−32]. In addition, Sugiura et al. [33] reported that pure glycerol
77 preparations produced methylglyoxal. However, until recently, there has been no research on the
78 influence of changes in the storage process and the use of glycerol as an excipient for
79 biopharmaceuticals. As an approved drug, thymopentin is the immunoactive center of thymopoietin II,
80 with an amino acid sequence of H-Arg-Lys-Asp-Val-Tyr-OH, and plays an important role as an
81 immune bidirectional regulator [34]. Thymopentin shows good clinical results in the adjuvant
82 treatment of chronic hepatitis, respiratory diseases, and malignancies [35]. However, like most
83 biopharmaceuticals, it is less stable in aqueous solutions, and the peptide bonds of Asp residues are
84 prone to cleavage, particularly in acidic environments [36]. Therefore, for the liquid formulation of
85 thymopentin, polyol excipients, such as glycerol and mannitol (Fig. 1), are often added as protective
86 agents to maintain their stability [3,4,10,37]. Thymopentin is a low-molecular-weight pentapeptide
87 that facilitates analysis of degradation mechanisms and sites. In this study, thymopentin was used as a
88 model drug to explore the stability of polyol pharmaceutical excipients (glycerol and mannitol) during
89 storage and use, their effects on biopharmaceutical stability, and the potential reactive sites of
90 thymopentin formulations. It is hoped that this study will increase the industry's attention to reactive
91 impurities arising from biopharmaceutical excipients during storage and use and demonstrate the
92 importance of maintaining low-temperature and oxygen-free storage conditions.

93 **2. Materials and Methods**

94 *2.1 Materials and chemicals*

95 Thymopentin was purchased from Nantong Feiyu Biological Technology Co., Ltd. (Nantong,
96 China). Glycerol and ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) of pharmaceutical
97 grade were purchased from Hunan Er-Kang Pharmaceutical Co., Ltd. (Changsha, China).

98 Guaranteed reagent-grade mannitol, monobasic sodium phosphate dodecahydrate, dibasic
99 sodium phosphate dihydrate, ferric chloride, sodium hydroxide, and formaldehyde were purchased
100 from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 3-Methyl-2-benzothiazolone

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101 hydrazone hydrochloride was purchased from BioDuly Co. Ltd. (Nanjing, China).

102 *2.2 Sample preparation*

103 Thymopentin formulations at a concentration of 1 mg/mL were prepared in sodium phosphate
104 buffer (pH 7.0, 10 mM) with 0.02% EDTA-2Na. The excipients mannitol and glycerol before and after
105 oxidative degradation were used as osmoregulators. These formulations were labelled as follows:
106 thymopentin formulations with undegraded mannitol (TP5-M0), thymopentin formulations with
107 degraded mannitol (TP5-M1), thymopentin formulations with undegraded glycerol (TP5-G0), and
108 thymopentin formulations with degraded glycerol (TP5-G1). All formulations were sterile-filtered
109 with a 0.22 μm Merck Millipore PES membrane filter (Darmstadt, Germany). The formulations (1.0
110 mL each) were then aseptically filled into presterilized 2-mL Schott vials (Lishui, China).

111 *2.3 Accelerated stability study*

112 The degraded glycerol and mannitol excipients were obtained by storage at 40 °C for 6 weeks.
113 The excipients were oscillated at the maximum speed for 1 min twice a week using a vortex oscillator
114 purchased from Sangon Biotech (Shanghai, China) to simulate the worst condition of excipients during
115 industrial production, that is, the excipients were fully exposed during each use. Different groups of
116 thymopentin formulations (TP5-M0, TP5-M1, TP5-G0, and TP5-G1) were stored at 40 °C. At each
117 time point (0, 1, 2, 4, and 8 weeks of storage) three vials of each formulation were used for reversed-
118 phase high-performance liquid chromatography (RP–HPLC) analysis. The 40 °C condition was
119 maintained in an LRH-250-II biochemical incubator purchased from Guangdong Medical Instrument
120 Factory (Guangzhou, China).

121 *2.4 Detection of aldehydes and reducing substances*

122 The contents of aldehydes and reducing substances in the polyol excipients and thymopentin
123 formulations were determined by phenol reagent spectrophotometry according to the Chinese
124 Pharmacopoeia (ChP) [34].

125 Detection of polyol excipients: The total reaction volume was 10 mL, and a 0.4 mL of
126 formaldehyde standard solution (5.0 μg/mL) was added to the positive control group ($\rho_{CH20}$=0.815
127 g/cm$^3$). In the experimental group, 0.2 g of glycerol and mannitol were added before and after
128 degradation, respectively. Then, 0.4 mL of the newly prepared 1% phenol reagent was added to each
129 tube and allowed to stand for 5 min. Subsequently, 1 mL of 0.5% ferric chloride solution was added
130 as the coloring reagent, followed by 5 mL of water. Finally, the volume was adjusted to 10 mL by using
131 methanol. The colors of the reaction systems of the polyol excipients before and after degradation were
132 observed.

133 Detection of thymopentin formulations Quantitative reactions were performed in 96-well plates. 134 The detection principle was the same as described above, and the total reaction volume was 200 µL. 135 First, a standard curve was obtained from a standard solution of formaldehyde (1 µg/ml), as shown in 136 Fig. S1 and the absorbance wavelength was 655 nm. The absorbance of TP5-M0, TP5-M1, and TP5-137 G0 formulations before degradation and after the 8-week stability test were detected at 655 nm. The 138 absorbance of the TP5-G1 formulations under the 40 °C stress test at weeks 0, 1, 2, 4, and 8 was also 139 detected at 655 nm under the same conditions ($n$=3). Finally, the aldehyde content was calculated 140 according to the standard curve and absorbance values.

141 *2.5 Gas chromatography with mass spectrometry (GC–MS)*

142 The structures of impurities in degraded glycerol were detected using Waters GCT Premier GC–143 TOF–MS (Milford, USA). GC conditions: type of injection, split; injection time, 0.5 min; capillary 144 column, Wax column (30 mm × 0.32 mm, 0.25 µm; Thermo Fisher Scientific Inc., USA). The column 145 temperature started at 60 °C, was held for 2 min, and then increased at a rate of 40 °C/min up to 120 °C 146 without retention, and then at a rate of 40 °C/min to 300 °C, which was held for 7 min. The temperature 147 of the inlet was 240 °C, the column flow was 1.84 mL/min, the split ratio was 10:1, and the carrier 148 gas was helium. MS conditions: ionization mode, EI; electron energy, 70 eV; the temperature of the 149 ion source, 200 °C; the temperature of quadrupole, 250 °C; scanning range, 35–450 amu.

150 *2.6 Reversed-phase high-performance liquid chromatography (RP–HPLC)*

151 The contents of thymopentin in different formulations were monitored using an Agilent 1260 152 HPLC (Agilent Technologies Inc., Karlsruhe, Germany) on a Supersil ODS column (4.6 mm × 250 153 mm, 2.5 µm; Dalian Elite Analytical Instruments Co., Ltd., Dalian, China) equipped with a DAD 154 G4212B detector. A gradient elution method was used for chromatographic separation, with 0.1% 155 formic acid (FA, *V/V*) in water as mobile phase A and 0.1% FA (*V/V*) in acetonitrile as mobile phase 156 B. The initial ratio of mobile phase B was 2%, which was increased to 25% over 35 min to elute 157 thymopentin, followed by 0.9 min of elution with 90% mobile phase B. Before use, the mobile phases 158 were filtered and degassed. The injection volume was 10 µL and the flow rate for the analysis was 0.5 159 mL/min. The column temperature was maintained at 30 ºC. The thymopentin and its degradation 160 products were monitored at 214, 220, 254, and 280 nm. The method validation data are presented in 161 Fig. S2 and Tables S1-S2.

162 *2.7 Liquid chromatography coupled with tandem MS (LC–MS/MS)*

163 The LC (1290 HPLC, Agilent Technologies, Inc., Karlsruhe, Germany) conditions for the 164 detection of degradation products were consistent with those of the RP–HPLC described in Section

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165 2.6, except that the injection volume was changed to 2 μL. The mass conditions (6460 Triple Quad
166 LC/MS, Agilent Technologies Inc., Karlsruhe, Germany) were as follows: ion source, ESI positive
167 mode; ion spray voltage, 5.0 kV; temperature, 350 °C; curtain gas, 5.0 L/min; nebulizer gas, 45.0 psi;
168 sheath flow rate, 10 mL/min; sheath temperature, 350 °C. First-order mass spectrometry was
169 performed using TIC full-ion scanning with a scanning range of 50–1000 amu. Based on LC–MS, a
170 relatively large number of degradation peaks (mainly the degradation products with peak areas >1%),
171 which require structural confirmation, were selected for tandem mass spectrometry detection. The
172 collision gas was nitrogen, the collision energy was set to 35 or 40 V, and the fragmentor was set to
173 135 V to obtain fragment ion peaks.

174 ## 3. Results and Discussion

175 ### *3.1 The color reaction results of degraded polyol excipients*

176 The results of the qualitative phenol reagent color reaction of the polyol excipients indicated that
177 glycerol before degradation (G0) and mannitol before and after degradation (M0 and M1) did not show
178 a blue–green color. However, the degraded glycerol (G1) was blue–green in the color reaction, which
179 suggested that there were aldehydes and reducing substances in the degraded glycerol sample, as
180 shown in Fig. 2. The content of aldehydes and reducing substances in the degraded glycerol exceeded
181 the requirement of no more than 10 ppm in the ChP and European Pharmacopoeia (EP); thus, this
182 excipient was unqualified after the stress test [34,38].

183 Glycerol and mannitol are polyol excipients with similar functional groups, as shown in Fig. 1.
184 However, they showed a dramatic difference in stability during storage and repeated use. The principal
185 reason why the stability of the two excipients differed so dramatically is probably that glycerol is a
186 liquid excipient, while mannitol is a crystalline solid, which showed much higher stability owing to
187 the restricted mobility and retarded exchange of oxygen on the surface. In addition, glycerol is highly
188 hygroscopic, and its oxidation is accelerated when the moisture content is increased. Thus, ChP and
189 EP require strict control of the moisture content of glycerol should be strictly controlled [34,38].
190 Mannitol, a crystalline sugar alcohol, is chemically stable and exhibits no hygroscopicity even under
191 extremely high relative humidity [39]. The implication for formulation development is that when
192 excipients with similar protective effects on biopharmaceuticals are selected, the more stable solid
193 excipients should take precedence over the liquid excipients owing to stability issues in long-term
194 manufacturing storage and use.

195 ### *3.2 Impurities in degraded glycerol*

196 To investigate the impurities produced by glycerol after degradation and their potential influence

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197 on biological drugs, GC–MS was used to detect glycerol and its degradation products. The reactive
198 impurities associated with thymopentin degradation are listed in Table 1. The representative mass
199 spectrum of glycerol impurity **4** (mean molecular weight=164 Da) is shown in Fig. 3, and the other
200 spectra of the glycerol impurities are shown in Fig. S3.

201 Impurity **1** is hydroxyacetone, which is presumed to be obtained from the dehydration of glycerol
202 and subsequent keto-enol tautomerization, as shown in Table 1. Impurity **2** is 2,3-epoxy-1-propanol
203 (glycidol), which is most likely formed by epoxidation via the $S_N2$ reaction between the vicinal diol
204 of glycerol.

205 1,2-Dihydroxypropionaldehyde (GLAD) and 1,3-dihydroxyacetone (DHA) are common
206 oxidation products in glycerol [29], and our thymopentin stability studies (see below) also suggest that
207 these two degradation compounds exist in degraded glycerol. However, they were not detected by the
208 GC–MS analysis. Instead, we identified two impurities, impurity **3** (a cyclic acetal) and impurity **4** (a
209 cyclic ketal), presumably formed by the reactions of GLAD and DHA with glycerol, respectively.
210 Owing to the large excess of glycerol, the reaction for GLAD and DHA could be driven nearly to
211 completion. Impurity **3** (CAS# 112401-29-3) has only been reported by Hibbert and Whelen [40] and
212 Fisher and Smith [41], but it was first reported to result from the storage of glycerol in our study.
213 Impurity **4** has not been reported previously in the literature. It has been reported that glycerol easily
214 reacts with acetone to form stable five-membered or six-membered ring compounds, depending on the
215 hydroxyl groups involved [42]. The stability of the cyclic acetal/ketal depends on strain energy. The
216 lower the strain energy, the more stable is the structure. Three types of strains contribute to the overall
217 energy of a cyclic acetal/ketal: torsional strain, angle strain, and steric strain [43]. Although the six-
218 membered ring is slightly more stable than the five-membered ring when considering the cycloalkane
219 ring strain, the product rings are not always suitable models of the transition states for some ring-
220 closure reactions [44]. For the reaction of glycerol and acetone, the selectivity for the formation of the
221 five-membered ring is better than that for the six-membered ring under most catalytic conditions
222 because of steric hindrance. The number of six-membered rings in the reaction products did not exceed
223 2%–3% under the most common acidic catalytic conditions [42,45,46]. The reaction was reversible,
224 and the acetal and ketal could be easily hydrolyzed back to GLAD and DHA. Therefore, the color
225 reaction and thymopentin stability results suggested that GLAD and DHA were present in the oxidized
226 glycerol.

227 Among the several new substances produced by glycerol degradation, hydroxyacetone (impurity
228 **1**), GLAD (hydrolysis product of impurity **3**), and DHA (hydrolysis product of impurity **4**) have
229 carbonyl groups, which are presumed to react directly with free amino groups in thymopentin through
230 a Maillard reaction [47].

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231 *3.3 The contents of aldehydes and reducing impurities in formulations and monitoring of thymopentin*
232 *contents by RP–HPLC*

233 To explore the influence of glycerol before and after degradation on the stability of
234 biopharmaceutical formulations, an accelerated stability test of thymopentin formulations was carried
235 out. In early excipient tests, we found that the aldehyde content in glycerol was high after degradation.
236 Therefore, the aldehyde content of the thymopentin formulations was determined during the stress test
237 (Fig. 4A). RP–HPLC results indicated changes in thymopentin content in each formulation from to 0-
238 8 weeks as shown in Fig. 4B. First, the effects of different excipients on thymopentin stability were
239 compared. No significant difference between TP5-M0 and TP5-G0 ($P$>0.05) was observed, indicating
240 that the protective effects of mannitol and glycerol on the stability of thymopentin preparations were
241 similar, and there were no obvious aldehyde impurities in the formulations even after degradation.
242 Regarding the effects on the stability of thymopentin in the same excipients before and after
243 degradation, no significant difference was observed between the TP5-M0 and TP5-M1 formulations
244 ($P$>0.05), which utilized undegraded and degraded mannitol, respectively.

245 However, thymopentin in the TP5-G1 formulations was significantly degraded ($P$<0.0001)
246 compared to TP5-G0, and the aldehyde content showed an unusual decrease. In the eighth week, the
247 thymopentin content was only 66.4% in the TP5-G1 formulations. The content of intact thymopentin
248 (red) and aldehydes (blue) in the TP5-G1 formulations are shown in Fig. 4B. It was found that the
249 trends of both thymopentin and aldehyde content were surprisingly consistent. First, the contents of
250 intact thymopentin and aldehydes in the TP5-G1 formulations were highest at week 0. Both the
251 thymopentin and aldehyde contents of TP5-G1 formulations showed a rapid decline during weeks 0-
252 2, from which it can be inferred that the aldehyde impurities in degraded glycerol reacted with
253 thymopentin. Finally, the aldehyde content tended to be stable at weeks 4−8, while the thymopentin
254 content decreased. This result indicated that during weeks 4−8, the decrease in thymopentin content
255 was primarily caused by the self-degradation of thymopentin during the 40 ºC stress test.

256 The chromatograms of all thymopentin formulations after 8 weeks of the accelerated stability
257 study are shown in Fig. 4C, and the peak at approximately about 13.0 min is the peak of thymopentin.
258 In general, the degradation of the TP5-M0, TP5-M1, and TP5-G0 formulations was similar, and only
259 three degradation peaks were observed. These three degradation products are caused by the instability
260 of thymopentin in aqueous solutions and are assigned to the self-degradation peak. However, a large
261 number of degradation product peaks were observed in the TP5-G1 formulations, indicating that
262 oxidized glycerol has a great influence on the stability of thymopentin formulations during storage. At
263 the same time, the three common self-degradation peak areas in the TP5-G1 formulations were
264 significantly higher than those in other formulations, indicating that degraded glycerol promoted the
265 production of these degradation products.

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266 Analysis of the degradation of thymopentin by HPLC further proved that some reactive impurities
267 produced in the degraded glycerol led to severe instability of the thymopentin formulations. Therefore,
268 researchers should pay more attention to changes in excipients during storage and use because they
269 may lead to a significant decline in the stability of biopharmaceutical preparations. In addition, TP5-
270 M1, after the accelerated stability test, had no such issues, suggesting that mannitol was more stable
271 than glycerol as an excipient.

272 *3.4 Characterization of the degradation products of thymopentin formulations by LC–MS/MS*

273 Because more degradation peaks were produced in the thymopentin formulation (TP5-G1)
274 containing degraded glycerol, this formulation was analyzed by LC–MS/MS to explore the
275 degradation mechanism of thymopentin. The structures of thymopentin and its major degradation
276 products (Table 2) were obtained using LC–MS/MS analysis, as shown in Figs. 5 and S4.

277 Table 2 shows that products **5** and **7** are formed by the degradation of thymopentin itself. Product
278 **5** is an isoAsp-containing variant of thymopentin and this impurity was also observed in other
279 formulations. Therefore, this degradation product is due to the degradation of thymopentin in solution
280 and has no direct correlation with the degraded glycerol. The Asp isomerization reaction has been
281 widely reported previously [48−50]. The specific process of Asp cyclization occurs through the attack
282 of N+1 main chain amide nitrogen on the Asp side chain carbonyl, resulting in a dehydrated aspartic
283 succinimide (Asu) intermediate, which is degradation product **11**. Then, the hydrolysis of the Asu
284 intermediate product **11** produces either isoAsp-containing product **5** or the Asp-containing starting
285 material thymopentin. The relative amounts of the isoAsp and Asp products formed depend on the pH
286 of the solution. Under neutral conditions, the iso-Asp product is usually favored [51]. Therefore, a
287 certain amount of product **5** was detected in the thymopentin preparation in this study. Theoretically,
288 the succinimide intermediate product **11** is unstable in aqueous solution; however, it was still detected
289 by LC–MS/MS. Product **7** is obtained by the cleavage of the peptide bond between the $Arg^1$ and $Lys^2$
290 residues. Products **5** and **7** further verify that the degradation peaks in all formulations are caused by
291 the degradation of thymopentin itself. Helm and Müller [52] reported that the liquid preparation of
292 thymopentin is unstable under acidic conditions, and the peptide bond of the Asp residue easily breaks
293 to form dipeptides and tripeptides. This study showed for the first time that the peptide bond between
294 Arg and Lys in a thymopentin liquid preparation was easy to break under neutral conditions.

295 Other thymopentin degradation products were generated via glycerol oxidation. Thymopentin
296 reacts directly with glycerol oxidation products, such as GLAD and DHA, to form products **8** and **9**.
297 GLAD and DHA were not detected in degraded glycerol but were probably the reversible hydrolysis
298 products of impurities **4** (an acetal) and **5** (a ketal) in the degraded glycerol, respectively. The other
299 degradation products that contain glycerol impurities are dehydration products, specifically, products

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300 **10**, **12**, and **13**. Among them, product **12** was formed by the dehydration of product **9a**, and similarly,
301 product **13** was obtained by the dehydration of product **8a**. Changes in the peak areas of products **8a**
302 and **13** and their conversion mechanisms are shown in Figs. 6 and 7, respectively. The main impurities
303 in glycerol oxidation products involved in the degradation of thymopentin are hydroxyacetone, GLAD,
304 and DHA. The products from the Maillard reaction of hydroxyacetone with the free amino group of
305 thymopentin were unstable and further dehydrated to produce the degradation product **10**. The health
306 risks of hydroxyacetone impurities in glycerol after dermal exposure have also been evaluated [53]. In
307 this study, it was found that this impurity could also react with free amino acids in biopharmaceuticals,
308 resulting in a decrease in the stability of the drug preparation, which affects its biological efficiency.

309 Degradation product **6** was speculated to react with formaldehyde in air. The guanidine group of
310 arginine has been reported to capture formaldehyde [54,55]. This is probably caused by impurities with
311 catalytic activity in degraded glycerol, which promoted the arginine guanidine group of thymopentin
312 to capture formaldehyde in the air, leading to further degradation of the thymopentin formulation.

313 In general, glycerol generates oxidation impurities, such as aldehydes and ketones (impurities **1**,
314 **3**, and **4**), during repeated use and/or improper storage. These impurities react with thymopentin to
315 produce various related thymopentin degradation products, such as products **8**, **9**, and **10**, as shown in
316 Fig. 8. There are two main reaction sites in thymopentin: the guanidine group on the arginine residue,
317 and the free N-terminal group. These two sites are prone to Maillard reactions with carbonyl impurities,
318 dramatically reducing the stability of thymopentin formulations in TP5-G1 and affecting their
319 biological activities. Therefore, researchers should raise awareness regarding the stability of excipients
320 during storage and use, especially for labile liquid excipients.

321 **4. Conclusions**

322 Overall, excipients are considered chemically inert and often fail to attract sufficient attention.
323 The trace active impurities produced in the process of repeated use and storage of excipients have a
324 great impact on biopharmaceuticals and deserve more attention. In this study, two active impurities
325 that have not yet been reported were found during glycerol storage. We then explored the mechanism
326 by which the degradation products of glycerol lead to a decrease in the stability of thymopentin
327 preparations. Furthermore, the physical state of polyol excipients with similar chemical properties
328 determines their stability during storage and use, with mannitol being much more stable than glycerol.

329 In summary, this study focused on impurities produced during the storage and use of
330 biopharmaceutical excipients. For manufacturers, more attention should be paid to the storage and use
331 environment (i.e., low temperature and anaerobic conditions). Moreover, the impurities and stability
332 of frequently used excipients should be regularly evaluated to ensure consistency between batches of

333 biopharmaceutical products. Finally, the results of this study indicate that when choosing excipients
334 with similar protective effects for biopharmaceuticals, solid excipients may be preferred over liquid
335 excipients owing to stability concerns during long-term manufacturing storage and use.

## 336 CRediT author statement

337 **Min-Fei Sun:** Methodology, Investigation, Formal analysis, Writing - Original draft preparation,
338 Writing - Reviewing and Editing; **Jia-Ning Liao:** Methodology, Investigation; **Zhen-Yi Jing:**
339 Investigation; **Han Gao:** Investigation; **Bin-Bin Shen:** Investigation; **You-Fu Xu:** Investigation; **Wei-**
340 **Jie Fang:** Conceptualization, Methodology, Writing - Reviewing and Editing, Funding, Supervision.

## 341 Declaration of competing interest

342 The author declares that there are no conflict of interest.

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471

472 ## Figure captions

473 **Fig. 1.** Structures of polyol pharmaceutical excipients: (A) glycerol, (B) mannitol.

474 **Fig. 2.** Spectrophotometry with phenol reagent.

475 **Fig. 3.** (A) Mass spectra of impurity **4** eluting at 7.4 min in GC-MS. (B) The structure of impurity **4**
476 and its mass spectrum fragments.

477 **Fig. 4.** (A) The contents of aldehydes at 0 and 8 weeks of each formulation group. (B) Degradation of
478 thymopentin in different formulations at weeks 0−8 by RP-HPLC (red); Changes in aldehyde content
479 in TP5-G1 formulations at weeks 0−8 (blue). (C) HPLC chromatogram of thymopentin formulations
480 at the 8th week. (TP5-M0 refers to thymopentin formulations with undegraded mannitol; TP5-M1
481 refers to thymopentin formulations with degraded mannitol; TP5-G0 refers to thymopentin
482 formulations with undegraded glycerol; TP5-G1 refers to thymopentin formulations with degraded
483 glycerol; TP5-0H refers to untreated thymopentin formulations; mean ± SD, $n$=3).

484 **Fig. 5.** (A) MS/MS analysis of thymopentin as a representative. (B) MS/MS spectrum of thymopentin.

485 **Fig. 6.** Change of peak area of products **8a** and **13** in weeks 0−8 of thymopentin formulations with
486 degraded glycerol (mean ± SD, $n$=3).

487 **Fig. 7.** Conversion mechanism of products **8a** and **13**.

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488 **Fig. 8.** Effect of polyol excipient stability during storage and use on the quality of the thymopentin
489 formulations.

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1 Table 1. GC–MS results of the main degradation products of glycerol and their potential reaction sources

| Impurities | Retention time (min) | Molecular weight (Da) | Structure | The potential reaction pathways |
|---|---|---|---|---|
| **1** | 3.4 | 74 | | |
| **2** | 4.2 | 74 | | |
| **3** | 7.2 | 164 | | |
| **4** | 7.4 | 164 | | |

2 * GLAD and DHA are direct oxidation products of glycerol. Their contents were extremely low, so they were not detected
3 by GC–MS, and their reaction products (impurities **3** and **4**) with glycerol were detected instead.

4 Table 2. Suggested structures of thymopentin degradation products in TP5-G1 formulations at week 8.

| Products | Retention time in HPLC (min) | Suggested structures of degradation products | Molecular weight (Da) |
|---|---|---|---|
| **5** | 14.7 | | 679.8 |
| **6** | 15.8 | | 691.8 |
| **7** | 16.7 | | 523.6 |

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| | | | |
|---|---|---|---|
| **8a/8b** | 17.4 | | 751.8 |
| **9a/9b** | 17.6 | | 751.8 |
| **10** | 17.7 | | 733.8 |
| **11** | 19.8 | | 661.8 |
| **12** | 23.0 | | 733.8 |
| **13** | 23.5 | | 733.8 |

5 TP5-G1: thymopentin formulations with degraded glycerol; RP–HPLC: reversed-phase high-performance liquid
6 chromatography.

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**A**

OH
HO OH

**B**

OH OH
HO OH
OH OH

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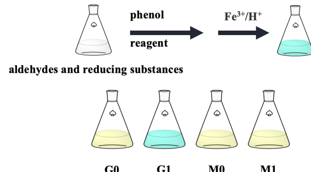

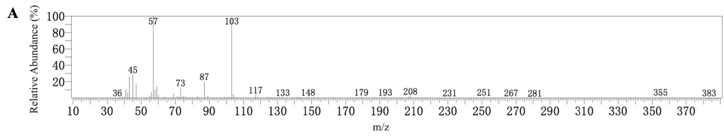


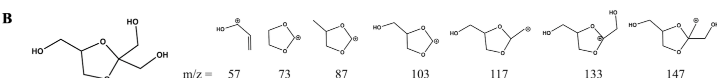

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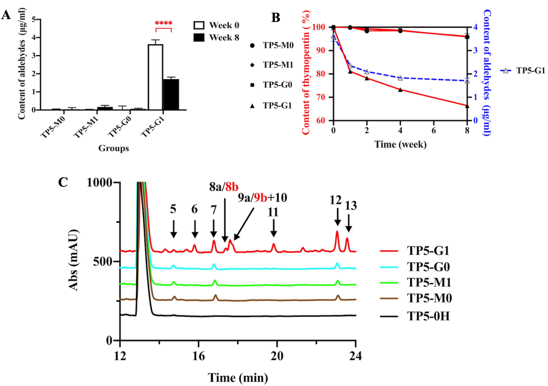


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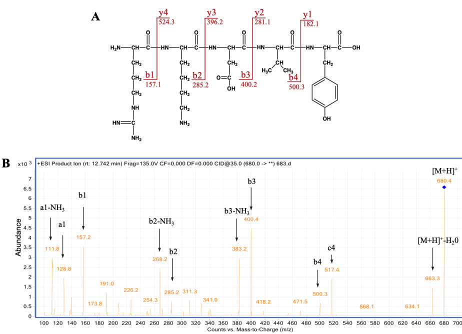


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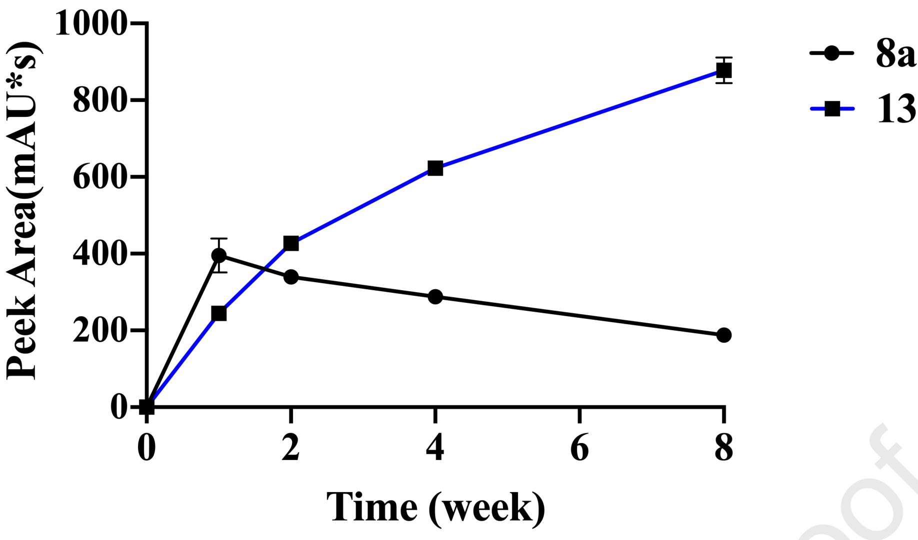


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HO OH N
**product 8a**

OH N

O N
**product 13**

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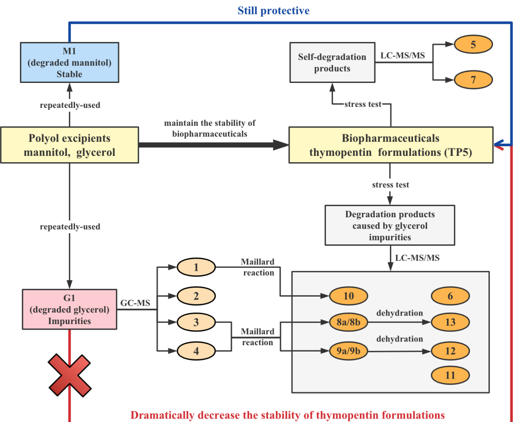


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## Highlights

- Unprecedented impurities in degraded glycerol were identified with GC/MS.
- Degradation of thymopentin due to glycerol degradation was determined using LC-MS/MS.
- Excipient stability affects biopharmaceutical formulation quality.

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