Dear Reviewer

We appreciate input. We carefully evaluated the valuable comments and adjusted the manuscript as necessary. Our point-by-point responses and the related adjustments are attached.

Review Report (Reviewer 3)
Round 2
Comments and Suggestions for Authors

The Authors made some corrections of the manuscript, which on my opinion didn't improve the overall scientific value of the manuscript. Some open remarks:

1. The manuscript lack "in vivo" verification of the optimal formulations as it is difficult to explain how this formulations e.g. S11 would behave in "in vivo" conditions in respect of the give results from table 5 (disintegration time – approx. 16 seconds and residence time approx. 118 minutes) and Figure 4 – less than 80% of API was released in 24 hour. These facts should be scientifically sound explained by the autors.

S11 would behave in "in vivo" conditions in respect of the give results from table 5 (disintegration time – approx. 16 seconds and residence time approx. 118 minutes)

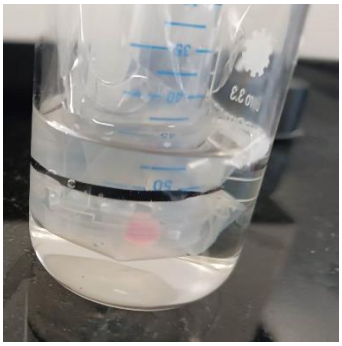
In the disintegration time test, the tablet was completely submerged in water on all sides. Conversely, in the residence time test, the tablet was initially moistened and then gently pressed against the mucosal surface beneath the tongue before being immersed in water. In this scenario, the tablet was surrounded by water on all sides, except for the side attached to the sublingual mucosa.

This attachment resulted in a strong interaction "stickiness" between the tablet and the mucin in the mucosal surface, primarily due to the mucoadhesive properties of certain polymers within the tablet's formulation. These interactions were notably strong and led to a significant delay in the tablet's disintegration when it was attached to the mucosal surface.

Figure 4 – less than 80% of API was released in 24 hour

In traditional drug release tests, the term 'drug release' typically refers to the process of the drug being released from the dosage form into the surrounding solvent. However, in our specific testing system, it involves measuring the release of the drug from the dosage form and its subsequent passage through a cellulose dialysis membrane, which covers the end of a plastic tube with a diameter of 2.5cm.

After a 22-hour period, it was observed that all the drug content had been passed from cellulose dialysis membrane. However, it's important to note that not all of this released drug passed through the dialysis tube. The passage of the drug through the cellulose membrane within the dialysis tube occurred at a slower rate compared to the initial drug release. Consequently, when we refer to drug release measurements, we are specifically referring to the amount of drug that successfully passes through the dialysis tube. Any drug that remains within the tube and does not pass through is not considered in this measurement.



According to our findings (resident time, release, and permeation), we expect that the new formulation, with a resident duration of 118 minutes, will have higher bioavailability than the typical sublingual tablet, which has a resident time of less than 5 minutes. This is the first phase in our research, which will be followed by in vivo testing in animals and compared to traditional sublingual Tablets in the future.

2. Authors should explain why only first the release profile in the 5.5 hours timeframe was used in elaboration of drug release kinetic mechanism in which only less than 50% of the incorporated drug was released.

We initially conducted the drug release profile analysis over a period of 22.5 hours, which served as the basis for establishing the kinetic release mechanism. However, following feedback from reviewer 4, we were advised to adjust the kinetic release model to fit a shorter duration of 5.5 hours.

In our initial kinetic release model fit, employing the DD solver program, we found that both the Makoid Banker model and the Peppas Shalin model adequately represented the drug release mechanism. However, upon reevaluation to meet reviewer 4's request, the adjusted analysis revealed that only the Peppas Shalin model accurately described the drug release mechanism.

Reviewer 4's feedback "The evaluation of the dissolution measurements must be repeated. The kinetic models must be fitted to the data recorded in the first 5 hours of the measurement, so it is highly likely that completely different fitting results will be obtained."

3. The authors should scientifically sound discuss the stability of the formulation (at least optimal) per see – stability of the API, which is chemically highly sensitive, in tablets on stress conditions. The stability study presented in Table 11 – stability of the API in water for 1 week or 3 months is not relevant nor for the formulation nor for the API.

we did stability in the simulated salivary fluid solution at 37 C for 24 hour (table 10)

All tests were conducted within a 24-hour timeframe, and the temperature did not exceed 37°C throughout all the steps.

B12 remained stable at pH 6.8 and 7.4 during this process.

Stability was confirmed at pH 6.8 through a stability test-step process. Initially, B12 was subjected to stability testing at 6.8 pH for 24 hours at 37°C, with UV results confirming the stability. The exact amount

of degradation was determined to be a loss of 672.195 µg (5.34%) where the remaining amount was higher than 90% which is acceptable as British pharmacopeia.[1]

In another stability test, a B12 stock solution (1000 µg/ml) was stored in simulated saliva fluid at pH 6.8 for 3 months, and the assessed recovery rate was approximately 96.456% (table 11). Consequently, the drug exhibits some stability but is not highly stable.

Furthermore, the optimal pH range for cyanocobalamin, the active pharmaceutical ingredient in our formula for prolonged storage, is 4-7 in solution. Our study aligns with this range, and the stabilization study for B12 can effectively use a phosphate buffer at pH 7.4, as employed in the permeation study, to stabilize the drug [2]

Also, these pH values were chosen based on previous studies, including drug release tests where release reached up to 100% in some formulations, and drug permeation studies conducted over 24 hours at pH 7.4 in a phosphate buffer at 37°C [3] [4].

Reference

1. Towers, M. Cyanocobalamin Tablets - British Pharmacopoeia 2023. *British Pharmacopoeia* 2023.
2. Schnellbaecher, A.; Binder, D.; Bellmaine, S.; Zimmer, A. Vitamins in Cell Culture Media: Stability and Stabilization Strategies. *Biotechnology and Bioengineering* **2019**, *116*, 1537–1555, doi:10.1002/bit.26942.
3. Mohamad, S.A.; Sarhan, H.A.; Abdelkader, H.; Mansour, H.F. Vitamin B12-Loaded Buccoadhesive Films as a Non-Invasive Supplement in Vitamin B12 Deficiency: In Vitro Evaluation and In Vivo Comparative Study with Intramuscular Injection. *Journal of Pharmaceutical Sciences* **2017**, doi:10.1016/j.xphs.2017.03.040.
4. Yang, Y.; Kalluri, H.; Banga, A.K. Effects of Chemical and Physical Enhancement Techniques on Transdermal Delivery of Cyanocobalamin (Vitamin B12) in Vitro. *Pharmaceutics* 2011, *3*, 474–484.

Concerning the stability of tablets (formulation)

The excipients utilized in this experiment were evaluated employing the following criteria:
1- Some excipients, (such as Microcrystalline Cellulose, Magnesium Stearate, PVP, HPMC), were used in several products on the market (as tablets), indicating that there is no interaction with the API.
2- For other excipients, we conducted a compatibility study for 1 month before using them to ensure that there is no interaction with the API, and based on the results, we selected the excipient where the assay was above 99% after one month.
It is important to conduct a stability study for the final tablets, as we plan to continue our study with an in vivo study, so we will include the tablet stability study before we conduct the invivo study.