Assay method validation of triamcinolone acetonide (TA) to support the investigation of TA-loaded nanoparticles

Validasi metode penetapan kadar triamsinolon asetonida (TA) untuk mendukung investigasi nanopartikel TA

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Abstract

The aim of this study was to develop the valid analytical method which used for the assay of triamcinolone acetonide (TA) in the investigation of TA-loaded nanoparticle formulations. High Performance Liquid Chromatography (HPLC) method was applied in this study by using an Econosil column, C18 10 µm, 250 x 4.6 mm (Alltech Associates Inc, PA, USA) as the stationary phase. The mobile phase consisted of a composition of acetonitrile (ACN) and 20mM phosphate buffer solution (pH 4.2) in the proportion of 50:50 v/v. The HPLC assay of TA was validated for selectivity, linearity, precision, recovery (accuracy), sensitivity and stability of TA during the assay. Results showed that the concentration of TA in the samples can be determined against the standard in the concentration range of calibration curve. The system precision and level of recovery were considered to be acceptable, and the method was selective and sensitive.

Key words: triamcinolone acetonide, assay, validation

Abstrak

Tujuan penelitian ini adalah untuk mengembangkan suatu metode penetapan kadar triamsinolon asetonida (TA) terkait dengan riset nanopartikel TA. Metode HPLC diaplikasikan dengan kolom Econosil sebagai fase diam. Sebagai fase gerak, diigunakan komposisi asetonitril: 2 nmM buffer fosfat (pH 4.2) dengan perbandingan 50:50. Validasi metode penetapan kadar TA ditetapkan dalam hal selektivitas metode, linearitas respon, ketelitian, ketepatan, sensitivitas dan stabilitas TA selama penetapan kadar. Hasil menunjukkan bahwa kadar TA dapat ditetapkan dalam rentang kurva baku yang dikehendaki. Ketelitian dan ketepatan metode dapat diterima, dan metode selektif serta sensitif dalam menetapkan kadar TA pada sampel.

Kata kunci: triamsinolon asetonida, penetapan kadar, validasi

Introduction

Recently, Triamcinolone acetonide (TA, MW: 434.5) with the structure as shown in Figure 1, has been extensively investigated in the treatment of a variety of ocular diseases associated with inflammation and neovascularisation (Jonas *et al.*, 2005; Rechtman *et al.*, 2003; Ciulla *et al.*, 2003). The angiostatic effect of TA in treating neovascularisation (Obata *et al.*, 2007), may provide potential for this agent to be developed as an antiangiogenesis agent, which could be useful in the treatment of a range of cancers.

TA is stable in its solid state, but it is unstable in aqueous-alkaline and ethanolic solutions. TA solution is more stable at pH values less than 5.5 and minimal degradation occurred at pH 3.4 (Gupta, 1982). As TA does not ionise, no information on the pKa of TA is available.
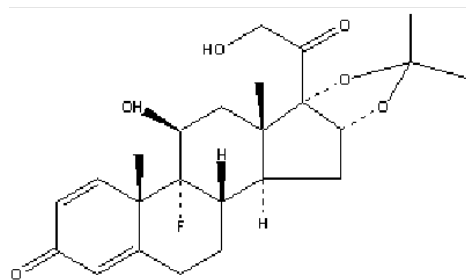
Figure 1. Structure of triamcinolone acetonide (Dollery, 1999).

By loading TA into nanoparticles (NPs), the nature of TA can be modified, leading to the improved delivery of the drug to the target site, which is facilitated by a controlled release. Besides, the NPs can protect TA from the unfriendly environment (Kreuter, 1994). Although TA loaded NPs have been initially investigated by Krause (1985), further research is needed using other biodegradable polymers and potentially superior methods, to improve the quality of NPs. To accommodate the further TA-loaded NPs investigation, a valid analytical method of determining TA needs to be developed.

Several publications have reported the novel HPLC assay for TA (Gupta, 1982; Derendorf *et al*., 1986; Matysova *et al*., 2003). A method established by Gupta (1982) was adopted in this research with some modification since it is stability-indicating with relatively short retention time.

Material and Methods
Materials
Micronised TA, batch number: 2196NMO0100421, chemical grade (Farmabios, Italy); Acetonitrile, HPLC grade (LabScan, Thailand); Orthophosphoric acid, Mw 98.00, BDH 'AnalaR', analytical grade, Min 85 % (BDH, Germany); Phosphate Buffer Solution pH 7.4 preserved by Sodium azide (PBS-NaN$_3$) in house production; empty poly-DL-lactide NPs (PDLLA-NPs), empty poly(lactic-co-glycolic acid) NPs (PLGA-NPs) and empty methoxy-polyethyleneglycol, poly(D,L, lactic-*co*-glycolic acid) NPs (mPEG-PLGA NPs), all were synthesised in house.

Methods
The TA HPLC method was adopted from a reported method (Gupta, 1982) with some modification. An Econosil column, C18 10 μm, 250 x 4.6 mm (Alltech Associates Inc, PA, USA) was used as the stationary phase. The mobile phase consisted of a composition of acetonitrile (ACN) and 20 mM phosphate buffer solution (pH 4.2) in the proportion of 50:50 v/v, filtered through a 0.45 μm membrane filter prior to use. The flow rate of 1.5 mL/min and the injection volume of 20 μL were used. The assay validation was conducted with TA standards dissolved in two media: the ACN:mobile phase 1:1 v/v (extracting media) and phosphate buffer saline pH 7.4 containing 0.05 % w/v sodium azide (PBS-NaN$_3$, release media). To obtain a maximum sensitivity, TA was measured at its wavelength of maximum absorption: 242 nm. The temperature of the system was maintained at 25 °C. The HPLC assay of TA was validated for selectivity, .linearity, precision, recovery (accuracy), sensitivity and stability of TA during the assay.

Selectivity
The peak purity of TA in two different media (ACN:mobile phase 1:1 and PBS-NaN$_3$) containing TA and degradation products were analysed to determine the selectivity of the assay.

Linearity
Linearity of the assay was determined in two types of media for TA loading and release study i.e. ACN:mobile phase 1:1 v/v and PBS-NaN$_3$, respectively. The calibration curve was constructed for each type of medium by plotting peak areas against the concentration of TA standard solutions.

Recovery (accuracy)
The accuracy of the HPLC assay was studied by examining the levels of recovery of TA in the presence of empty NPs made of three different polymers by the same method used for TA loading determination. The recovery was calculated by comparing the actual TA concentration detected with the initial concentration.
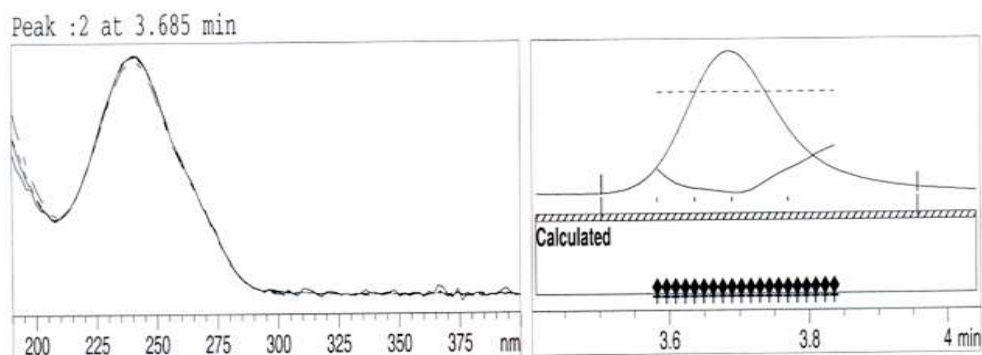
-> The purity factor is within the calculated threshold limit. <-

Figure 2. Purity factor of TA peak on HPLC assay.

Table I. Precision of the HPLC method for TA determination

Analytes	Average of AUC (mAu*sec)	RSD (%)
1.03 µg/mL of TA solution in ACN: mobile phase 1:1	27.17	1.25
30.90 µg/mL of TA solution in ACN:mobile phase 1:1	764.33	1.61
0.53 µg/mL of TA in PBS-NaN₃	13.12	0.53
5.27 µg/mL of TA in PBS-NaN₃	130.00	0.08

Sensitivity

Blank solvents were injected six times consecutively into the HPLC column and the standard deviation of average noise levels in the expected retention time were determined. For the TA loading determination assay, blank solvent was defined as the loading media after being filtered from blank NPs, while in the release study, blank solvent was the PBS-NaN₃. The theoretical LOD and LOQ were then calculated by the following formula:

$$LOD = \frac{standard\ deviation\ of\ noise\ levels}{slope\ of\ height\ versus\ concentration\ of\ calibration\ curve} \times 3$$

$$LOQ = \frac{standard\ deviation\ of\ noise\ levels}{slope\ of\ height\ versus\ concentration\ of\ calibration\ curve} \times 10$$

Stability assay of TA in aqueous environment

The TA solutions with known concentrations were placed in 1.5 mL vials for sampling and kept under the experimental temperatures. Samples were taken at 3, 6, 9, 24, and 72 hours after initial incubation and determined by the HPLC assay. The potency of TA was determined according to the following formula:

$$Potency\ of\ TA\ (\%) = \frac{peak\ area\ of\ TA}{total\ peak\ areas\ of\ TA\ and\ degradation\ products} \times 100$$

Results and Discussion

To determine the TA concentration, an HPLC assay developed by Gupta (1982) was adopted with some modification. Selectivity in the HPLC assay development was assessed to determine that the peaks can be well-separated (Figure 2). The retention time of TA and its degradation product in release medium (PBS-NaN₃), which is speculated to be 6β-hydroxyltriamcinolone (Sieh, 1982), were approximately 3.7 and 2.9 minutes respectively, showing that they were completely separated (Figure 3.(c)).
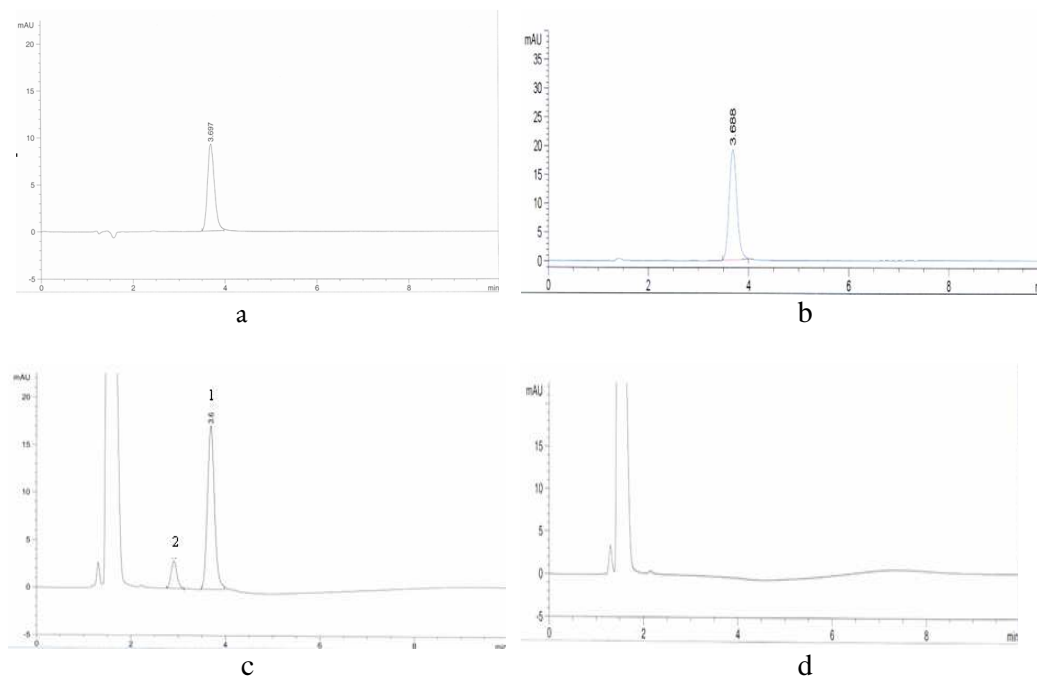
a

b

c

d

Figure 3.

    (a) Typical HPLC chromatogram of TA standard in ACN:mobile phase 1:1.

    (b) Typical HPLC chromatogram of TA extraction from loaded NPs in ACN: mobile phase 1:1.

    (c) Typical HPLC chromatogram of TA (peak 1) and degradation product (peak 2) in PBS-NaN₃ at 37 °C over 24 hours.

    (d) Typical HPLC chromatogram of PBS-NaN₃ (release medium).

Table II: Recovery of TA in the presence of empty NPs in mobile phase

Parameters	Empty PDLLA NPs	Empty PLGA NPs	Empty mPEG PLGA
Recovery (%)	99.15 ± 2.79	99.69 ± 1.97	99.91 ± 2.41
Slope	0.9849	0.9941	0.9921
Correlation coefficient	0.9999	0.9999	0.9999
95 % CI slope	0.9738 - 0.9960	0.9813 - 1.0070	0.9798 – 1.0045
95 % CI Y-intercept	-0.5187 to 0.5050	-0.6809 to 0.5036	-0.5961 to 0.5427

In the release medium, the buffer substances and the sodium azide (NaN₃, a preservative) eluted faster than TA (at 1.6 minutes), suggesting that those compounds did not interfere with the TA analysis.

    Correlation coefficient r^2 was calculated for the calibration curve of area under the curve (AUC) against the TA concentration in both media (Figure 4). The detector's response was found to be linear with r^2 of 0.9999 and 1 for ACN:mobile phase 1:1 media and PBS-NaN₃, respectively. This suggests that the concentration of TA in the samples can be determined against the standard in the concentration range of calibration curve.
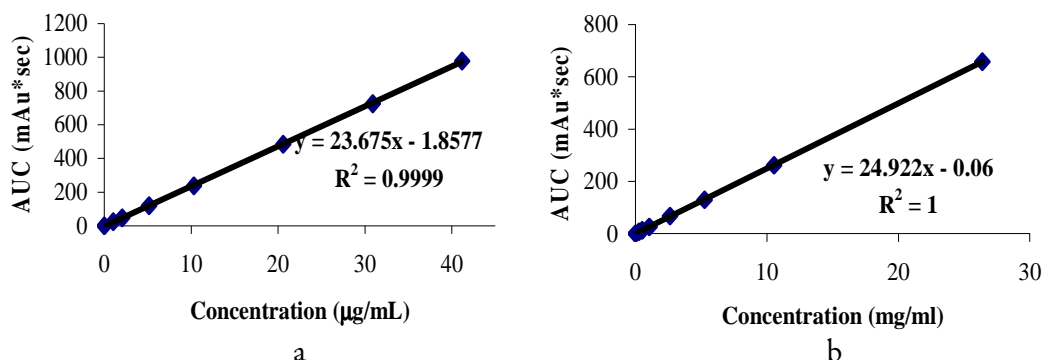
Figure 4. Standard curve for TA in ACN:mobile phase 1:1 (a) and TA in PBS-NaN3 (b).

Table III. LOD/LOQ of the HPLC assay for TA determination

	LOD (μg/mL)	LOQ (μg/mL)
Loading determination:		
- PDLLA NPs	0.090	0.299
- PLGA NPs	0.094	0.315
- mPEG PLGA NPs	0.118	0.394
Release study:	0.053	0.180

Precision for the HPLC assay is shown in Table I. The relative standard deviation (RSD) of the injections was obtained below the nominal acceptable level of 2 %, indicating that the injection of assay was precise for TA analysis.

The recovery of TA was investigated for determination of TA loading to assess the interference of the NP matrix in the assay. The results are presented in Table II.

In the presence of empty PDLLA NPs, the 95 % confident interval (CI) of the slope was 0.9738-0.9960, not including 1 (Table II). It indicates that the TA was not totally recovered by the assay method in the presence of the PDLLA matrix. However, the average of recovery was in the value of 99.69 $\pm$ 1.97 %, with a slope and the correlation coefficient of 0.9849 and 0.9999, respectively. The 95 % CI of Y-intercept goes through zero, therefore, this level of recovery was considered acceptable. The recovery levels in the presence of empty PLGA NPs and empty 4.8 % mPEG-PLGA

NPs revealed 95 % CI of slope including 1 and 95 % CI of Y-intercepts going through zero. These data indicate that the assay is unbiased and produces a satisfactory recovery. The recovery of TA in the release media was not carried out since there was no extraction method prior to TA determination by HPLC in the release study.

In order to assess the sensitivity of the assay, the theoretical LOD and LOQ were determined both in the loading determination and in the release study (Table III).

To determine whether the stability of TA in the aqueous environment could be maintained during the assay, an investigation of the stability of TA in the aqueous medium was carried out. In this study, the potency of TA in aqueous solutions was determined over 72 hours after storage at three different experimental temperatures: 4 °C, 25 °C, and 37 °C. The data of stability study are shown in Figure 5 and 6.
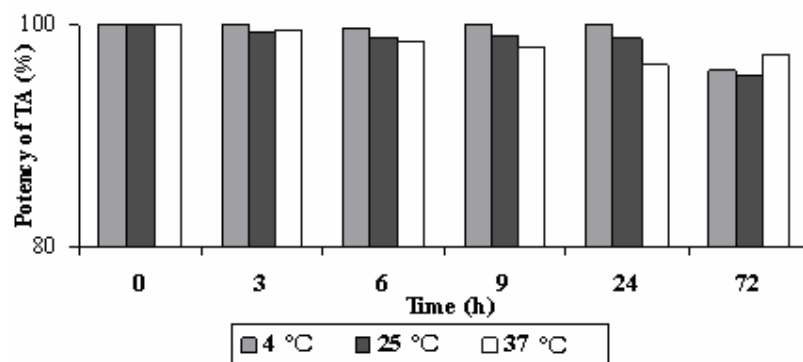
Figure 5. Potency of TA over 72 hours. Samples with a known concentration of TA in water were kept under experimental temperatures of 4 °C, 25 °C, and 37 °C for 72 hours.
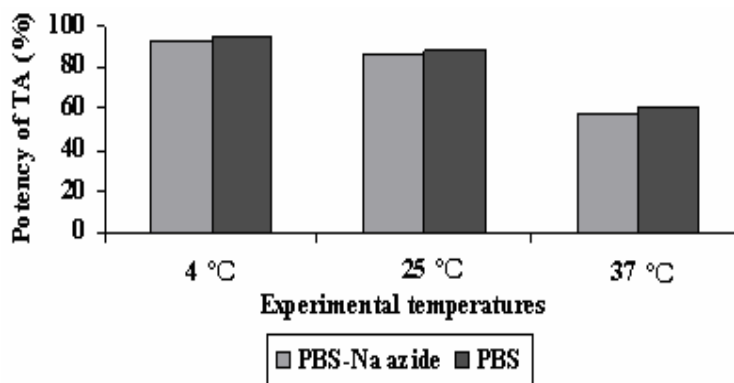

Figure 6. Potency of TA at 72 hours. Samples with a known concentration of TA in PBS and PBS–Na azide (PBS-NaN3) were kept under experimental temperatures of 4 °C, 25 °C, and 37 °C for 72 hours.

TA stability in the extraction medium (ACN:mobile phase 1:1 v/v) was analysed using the HPLC assay. The results showed that TA degraded slightly faster at higher temperatures (Figure 5). Although Gupta (1982) reported that TA is likely to be unstable at pH above 5.5 in an aqueous medium, a slow process of the degradation was also observed in the aqueous medium with pH approximately 7.

The stability of TA in the release medium was assessed at 4 °C, 25 °C, and 37 °C using HPLC assay. To investigate whether the stability of TA is affected by microbial activity, the study was conducted in the release media, with and without a preservative. It was found that the change of potency of TA appeared to be insignificant affected by the inclusion of preservative in the release media over 72 hours (Figure 6). It may suggest that microbial activity does not influence the degradation process of TA.

In the release medium, TA was degraded the fastest at 37 °C, as predicted. After 72 hours storage at 37 °C, the degradation was increased dramatically up to 43 %. A substantial decomposition of TA was found at room temperature, with TA potency reduced to 85 % over 72 hours, while at 4 °C the TA showed less than 10 % degradation (6.4 %).

Conclusions

The assay method of TA showed linear response of the detector suggesting that the concentration of TA in the samples can be determined against the standard in the concentration range of calibration curve. The precision and level of recovery were considered to be acceptable, and the method was selective and sensitive.

Acknowledgements

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