

Application of Ethanolic Potassium Hydroxide and Glacial Acetic Acid in the Spectrophotometric Determination of Prednisolone

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The use of tetrazolium salt as a colorimetric reagent has been widely applied for steroid determination. It is also the basis of some official assays¹⁻³. It is well known that corticosteroid containing α -ketol group possesses the reducing properties. Upon reduction, tetrazolium salt yields a deep red coloured formazan³⁻⁴. A colorimetric method for determination of 17-ketosteroids with tetrazolium salts in the presence of tetramethyl ammonium hydroxide (TMAH) was first suggested by Mader and Buck⁵. Factors affecting the method had been studied and made some improvement published by a number of workers⁶⁻¹². According to those modified methods and following examined we found that there were still disadvantages of high and continuously varying blank readings. Also it is time consuming in the manipulation. Reproducible result can't so easily be obtained. Factors such as oxygen, light, temperature and base can cause the variation in the rate of colour development^{6, 12}. For the purpose of trying to develop more reliable assay method, the advantage of using ethanolic potassium hydroxide instead of TMAH was investigated in this study. We found that the ethanolic potassium hydroxide can act as TMAH during the formazan formation and the addition of glacial acetic acid, or formic acid, at ten minutes after the reaction occurred can stabilize this colour reaction. A coloured formazan is stable in this condition under 24 hours observation. It is not affected by the daylight and the reproducible result could be obtained. The colour of absorption is proportional to the concentration of prednisolone in the range of 10-800 mcg. This assay method produces greater accuracy in testing and more rapid result.

Experimental

Apparatus

The Coleman (III) Spectrophotometer (Hitachi perkin-Elmer), MRK Model 10-250 AH 21 pH meter, and Thomas Hover capillary melting point apparatus were used in this work.

Materials

Tetrazolium blue—E. Merck

Tetramethyl ammonium hydroxide solution 10%—E. Merck

Citric acid, Oxalic acid—Cheng Hsin Tang Chemicals Co.

Benzoic acid, Propionic acid—Kanto Chemical Co., Inc. (Cica)

Formic acid—Nippon Shiyaku Kogyo K. K.

Potassium hydroxide, Phosphoric acid—Hayashi Pure Chemical Industries Ltd.
Sulfuric acid, Nitric acid, Hydrochloric acid—J. T. Baker Chemical Co.
Glacial acetic acid—Kokusan Chemical Works, Ltd.

Reagents

Aldehyde free alcohol—It was prepared according to the procedure of U. S. P. method¹⁰.

Tetrazolium blue solution contains 5 mg of tetrazolium blue per milliliter of aldehyde free alcohol was freshly prepared for each series of determinations.

A 1.0 N ethanolic potassium hydroxide is stored in a brown polyethylene bottle at room temperature. The 0.25 N, 0.50 N, and 0.75 N ethanolic KOH solution are prepared by appropriate dilution of the 1.0 N ethanolic KOH with aldehyde free alcohol.

The 0.5, 1.0, 2.0 and 5.0 N acetic acid of aqueous solution were obtained by dilution of glacial acetic acid with distilled water.

The concentration of inorganic acids, sulfuric acid, nitric acid, hydrochloric acid, phosphoric acid, used in this study were 1N of aqueous solution. 0.1M oxalic acid, 0.1M citric acid of aqueous solution and 0.1M benzoic acid of ethanolic solution were applied in this experiment.

Standard Preparation

Prednisolone solution was prepared by dissolving accurately weighed prednisolone U. S. P., previously dried at 105°C for 3 hours, in aldehyde free alcohol containing 1mg per milliliter. Then dilute the stock solution with aldehyde free alcohol to varied concentration from 50 mcg/ml. to 0.5 mcg/ml.

Assay

Pipet 20ml of prednisolone solution with various concentrations into the 25ml glass-stoppered conical flask, respectively. 20ml of aldehyde free alcohol in a similar flask was served as a reagent blank. To each of these flasks add 2ml of 0.5N ethanolic potassium hydroxide and 2ml. of tetrazolium blue solution, mix, and allow to stand for ten minutes then add 1ml. of glacial acetic acid immediately, read absorbance in a glass cell with 1cm light path at 525 m μ .

Results and Discussion

Absorption curve—The color was developed as directed under Assay Procedure, in this way the absorption curve was examined with 200mcg prednisolone in 25ml of total reaction mixture, corrected by the reagent blank. The result of this observation was presented in Fig. 1. It was noted that the maximum observation was the same as the U. S. P. method at 525 m μ . The same result was obtained from the prednisolone solution and reagent blank, corrected by aldehyde-free alcohol blank, as indicated in Fig. 2.

Standard curve—The absorbance of the color developed was proportional to the concentration of prednisolone, when assayed as mentioned above for a series of 25ml. reaction mixture containing various concentration of prednisolone, shown in the Fig. 3. The absorption of colour proportional to concentration of prednisolone in the 10-800mcg range exam-

ined. The result was plotted and show in the Fig. 4. The maximum deviation of the absorbance was 0.6% from the mean of 62 analyses examined.

Comparison of the color development between ethanolic KOH and TMAH—The color development using ethanolic potassium hydroxide as a base to accelerate Formazan reaction by U. S. P. method was faster and more intense than that of tetramethylammonium hydroxide. It was illustrated in Fig. 5, 6. The use of ethanolic KOH gives a low absorbance of reagent blank valued between 0.015-0.018, while the latter about 0.090-0.120.

Effect of KOH concentration on Formazan reaction—The choice of the final concentration of potassium hydroxide was based on the data of Table I. It appears that full colour development is reached at a final concentration of 4×10^{-2} N KOH. This experiment was determined by two analysts and same results were obtained.

Effect of glacial acetic acid—Using ethanolic KOH as a base instead of TMAH still existed its problem. Without the addition of glacial acetic acid slight turbidity was appeared in some reaction mixture while stand over 15-30 minutes. The cause of this hindrance to the determination might be the presence of carbon dioxide and aldehyde trace which produced potassium carbonate precipitation and interfered with the stability of the absorbance. The continuous color development caused by other factors both in reaction mixture and reagent blank could be eliminated by the addition of glacial acetic acid. The absorbance were constant for over 6 hours examined. Even though 24 hours passed the colour intensity decreased only 0.3%. The effect of the volume of glacial acetic acid was determined, and 1ml. of glacial acetic acid was the most acceptable quantity. It was indicated in the Table II.

Rate of colour development—Data of Fig. 7 show the typical time course of the reaction at room temperature. From the observation there was a rapid colour development in the reagent blank after 10 minutes. Rechnagel and Litteria¹² reported that the oxidation of ethanol occurred to an appreciable extent in the presence of tetrazolium blue, and the kinetics of the reaction imply that there was an initial rapid reduction of the tetrazolium blue by the steroid, accompanied by a further reduction of the dye by oxidation products of the ethanol. According to the data of Fig. 8 the rate of colour development decreased slightly after ten minutes, a point where it had the maximum absorbance. Thus the right time to add glacial acetic acid, 10 minutes after prednisolone reacted with tetrazolium in the presence of 0.5N KOH, was decided.

Effect of various acids on Tetrazolium blue reaction—Rechnagel and Litteria¹² also reported that the final alkalinity was shown not to be a factor to give aberrant results, but in the presence of tetrazolium blue alcohol was. In order to find out the reagent to prevent the oxidation of the ethanol in the presence of tetrazolium blue, four kinds of inorganic acid and six kinds of organic were tested. From Table III it appeared that inorganic acids didn't have this ability. When inorganic acid met potassium hydroxide in the reaction mixture or ethanolic solution a white precipitate was produced. It was assumed a potassium salt. In the case of nitric acid the white precipitate was separated and examined. The m.p. of it was 333°C as that of potassium nitrate. Among six kinds of organic acid we found that glacial acetic acid and formic acid were the most ideal reagents for this purpose.

In this study, we made the comparison between the use of actinic glass ware and the use of clear glass. The result showed no any difference when the color developed with base

and the color reagent. The reaction mixture exposed to the daylight showed no significant change in the absorbance values even though standing for over 6 hours. In view of these observation, it indicated that the daylight need not to be excluded from the laboratory when the reagents were added to the solutions and the spectrophotometric measurements are being made.

Final Normality of KOH	Optical Density at 525 mu
2×10^{-3} (+2ml. 0.25 N KOH)	0.455
4×10^{-3} (+2ml. 0.50 N KOH)	0.465
6×10^{-3} (+2ml. 0.75 N KOH)	0.455

Table I. Effect of KOH concentration: Read against aldehyde free alcohol as blank. 160mcg prednisolone in 25ml. of total reaction mixture used.

Table II. Effect of the Volume of Glacial Acetic Acid

Volume of Glacial Acetic Acid	Optical Density at 525 mu
1ml.	0.435
2ml.	0.435
3ml.	0.435

Condition: 160mcg prednisolone, 2ml. tetrazolium blue and, 2ml. 0.5N ethanolic KOH, were incubated for 10 minutes at room temperature, and glacial acetic acid was added immediately, then aldehyde free alcohol was added to make final volume 27ml. Read against reagent blank.

Table III. Effect of Various Acids on Tetrazolium Blue Reaction

Acid	Optical Density at 525mu			pH of final mixture
	5 minutes	30 minutes	6 hours	
1 N H_2SO_4	ppt.	/	/	>14
1 N H_3PO_4	ppt.	/	/	>14
1 N HCl	0.018	0.023	ppt.	>14
1 N HNO_3	ppt.	/	/	>14
0.1 M citric acid	turb.	/	/	>14

0.1 M oxalic acid	turb.	/	/	>14
0.1 M benzoic acid	0.018	turb.	/	>14
propionic acid	0.017	0.017	0.015	7.2-7.3
formic acid	0.017	0.017	0.017	4.2
glacial acetic acid	0.018	0.018	0.018	5.8-6.0
5.0 N acetic acid	0.018	0.018	0.019	7.2
2.0 N acetic acid	0.018	0.018	0.019	>14
1.0 N acetic acid	0.018	0.018	0.019	>14
0.5 N acetic acid	0.020	0.030	turb.	>14

Condition: 20ml. aldehyde free alcohol, 2ml. tetrazolium blue and 2ml. KOH, were mixed after ten minutes, 1ml. acid was added and read absorbance at 525nm. Aldehyde free alcohol only was used as the blank.

Summary

- (1) The use of 0.5N ethanolic potassium hydroxide instead of a 1:10 dilution of tetramethylammonium hydroxide was available for the spectrophotometric determination of prednisolone. It saved the manipulation time.
- (2) The suffering inconvenience of the further color development in the other method could be avoided by adding glacial acetic acid in this method. Adding of glacial acetic acid or formic acid prevents the oxidation of ethanol in the presence of tetrazolium blue and makes the reaction mixture having stable quantitative colour development. Also, the reproducible result could be easily obtained.
- (3) The precise time must be taken when glacial acetic acid was added to the reaction mixture. This modified method wasn't affected by the light, and yielded a low blank and higher sensitive colour development in sample solution. The fast and the ease with which this procedure can be applied would make it to be the method of choice.

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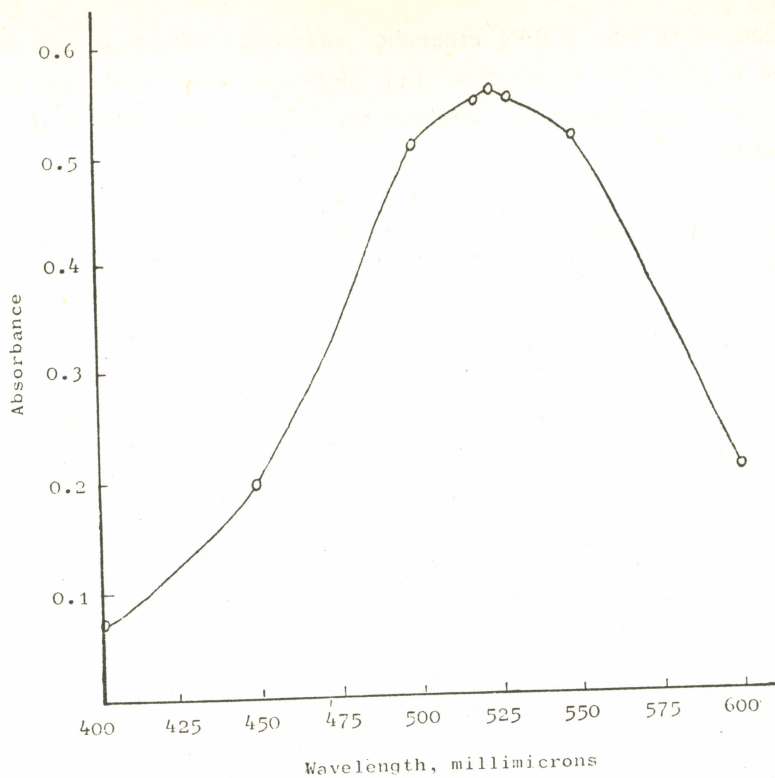


Fig. 1. Absorption curve—200 mcg prednisolone in 25ml. reaction mixture. Read against the reagent blank.

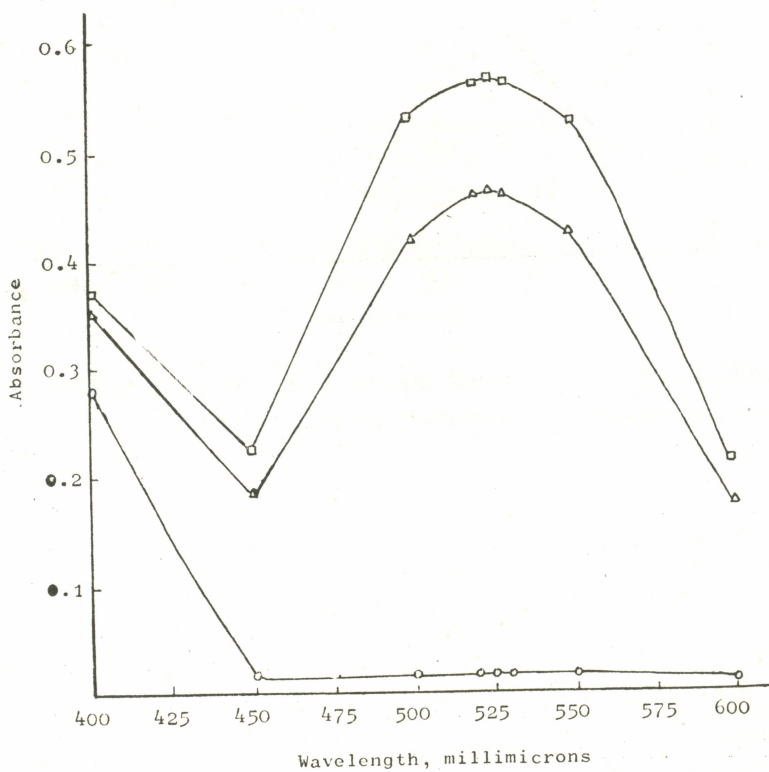


Fig. 2. Absorption curve—2ml. tetrazolium blue solution was added to the pred-

nisolone solution, then 2ml. of 0.5N ethanolic potassium hydroxide was added and mixed, allowed to stand for 10 minutes, 1ml. of glacial acetic acid, was added and mixed again, read absorbances at interval wavelength. corrected with aldehyde free alcohol blank.

- ; reagent blank
- △—△—; 160mcg prednisolone in 25ml. total reaction mixture
- ; 200 mcg prednisolone in 25ml. total reaction mixture

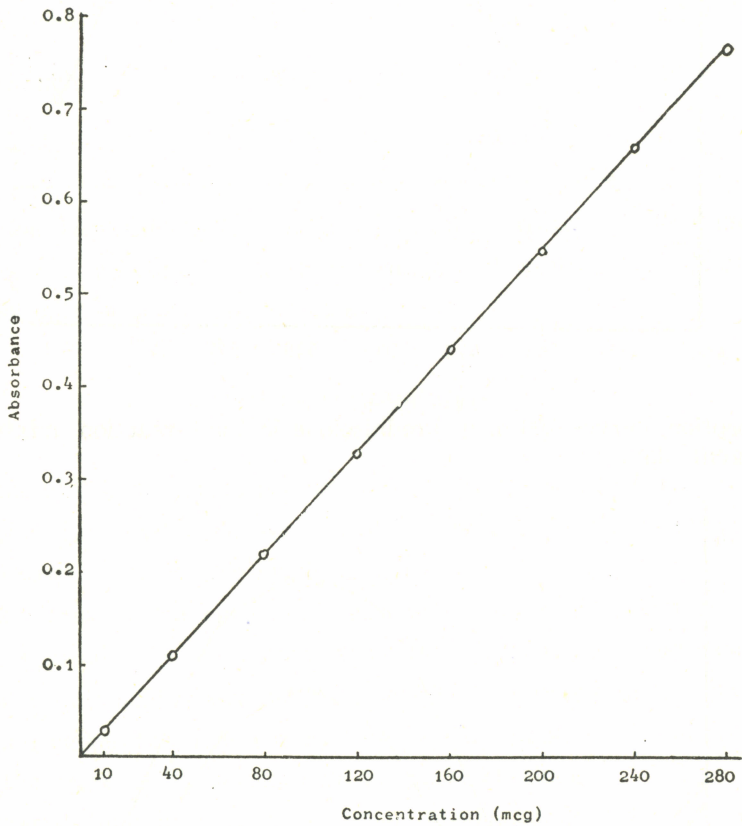


Fig. 3. Standard curve—The varied amount of prednisolone in 25ml. total reaction mixture were examined, corrected with the reagent blank

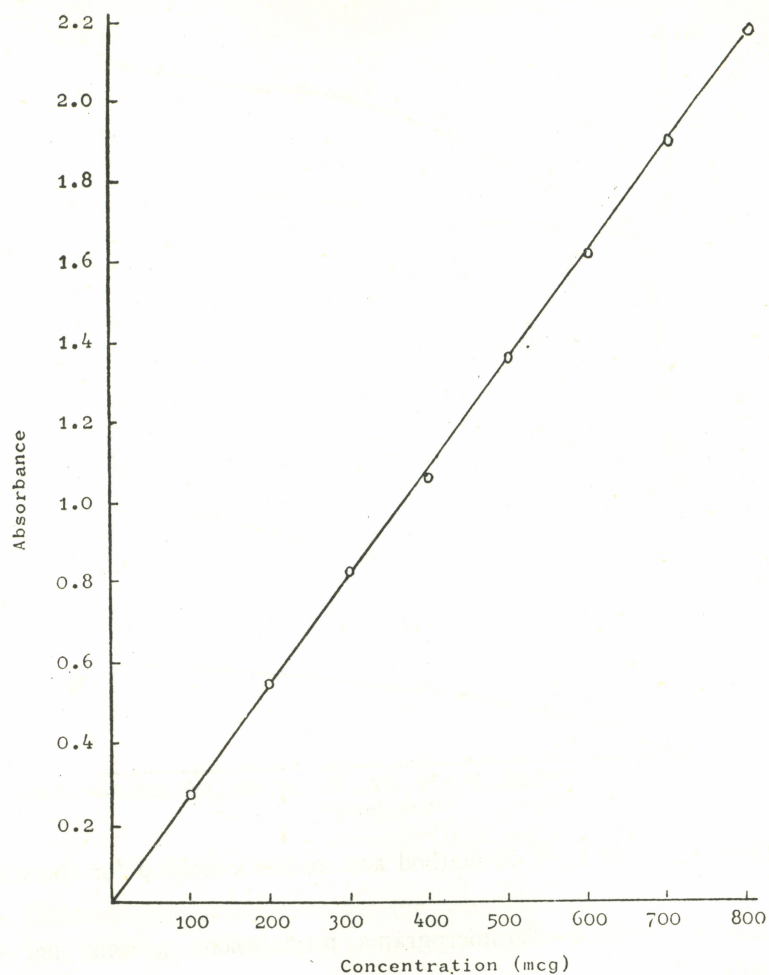


Fig. 4. The absorption of colour was propotional to concentration of prednisolone in the 10-800 mcg range examined.

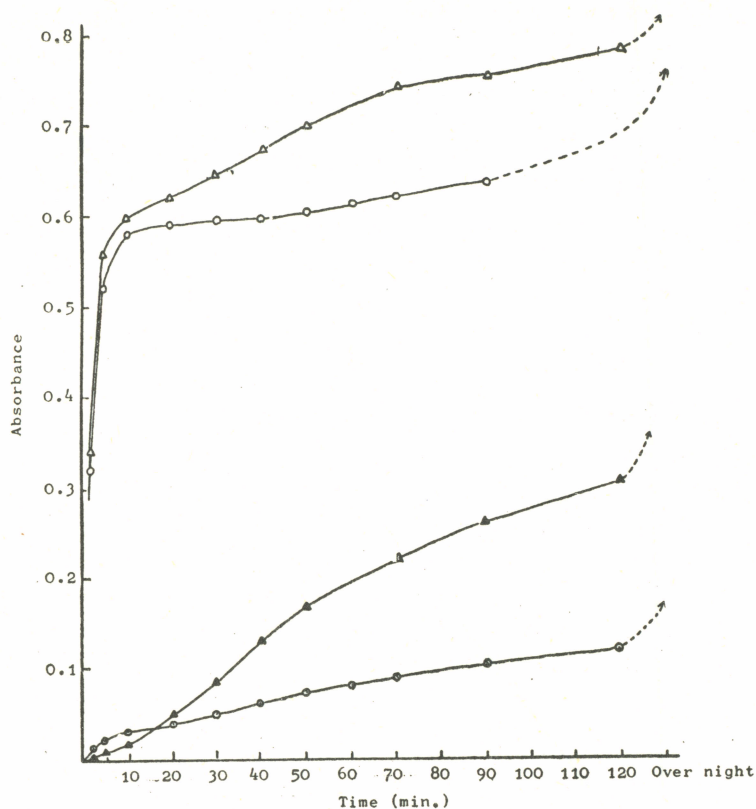


Fig. 5. Comparison of U. S. P. method and author's method for the colour development.

△-△-△; author's method—200 micrograms prednisolone present per colorimeter tube.

▲-▲-▲; author's method—reagent blank

○-○-○; U. S. P. method—200 micrograms prednisolone present per colorimeter tube

⊙-⊙-⊙; U. S. P. method—reagent blank

author method—condition, as in assay procedure with the exception of glacial acetic acid

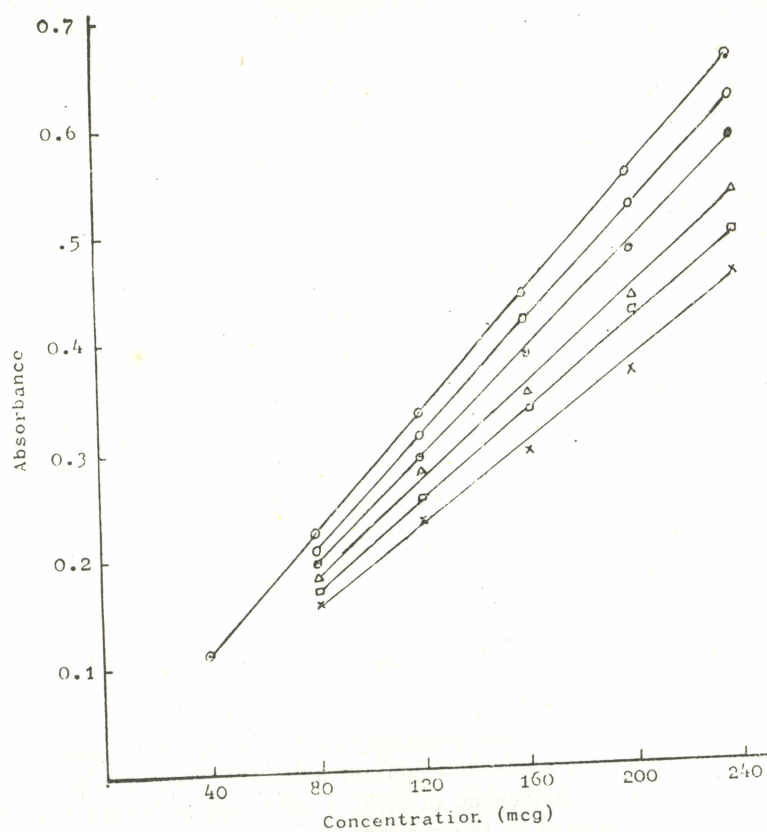


Fig. 6. Different incubation time for the color development.

⊙—⊙—⊙; Author method—incubation time 10 min.
 ○—○—○; U. S. P. method—incubation time 10 min.
 ●—●—●; U. S. P. method—incubation time 35 min.
 △—△—△; U. S. P. method—incubation time 50 min.
 □—□—□; U. S. P. method—incubation time 70 min.
 ×—×—×; U. S. P. method—incubation time 90 min.
 *room temperature

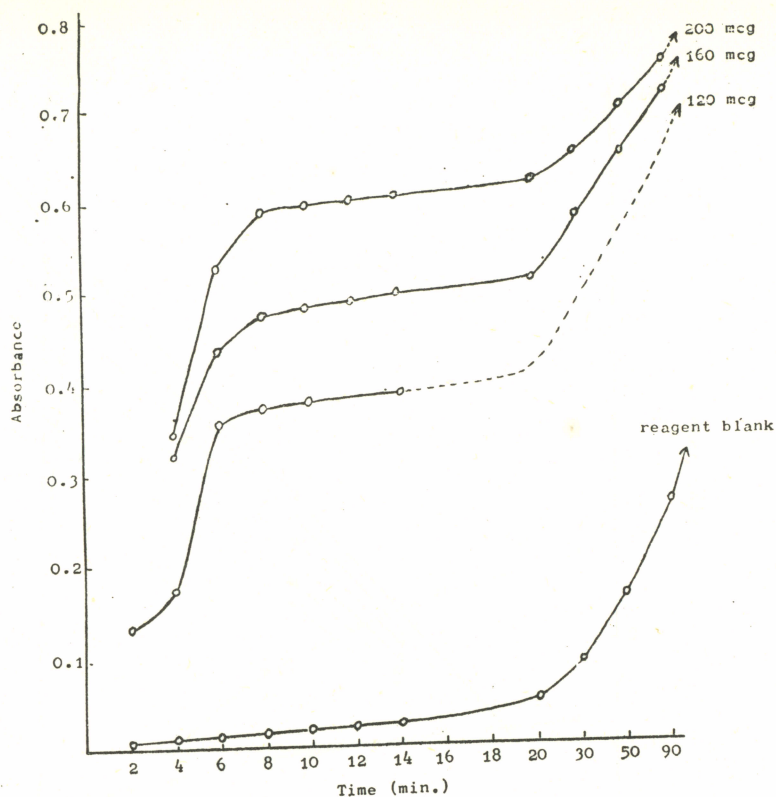


Fig. 7. Time course of the reaction—In the absence of glacial acetic acid, the reaction mixture's were measured against aldehyde free alcohol as blank. Figures denote micrograms prednisolone in 24ml. total mixture. It indicated that continuously various reading presented in the reagent blank with time.

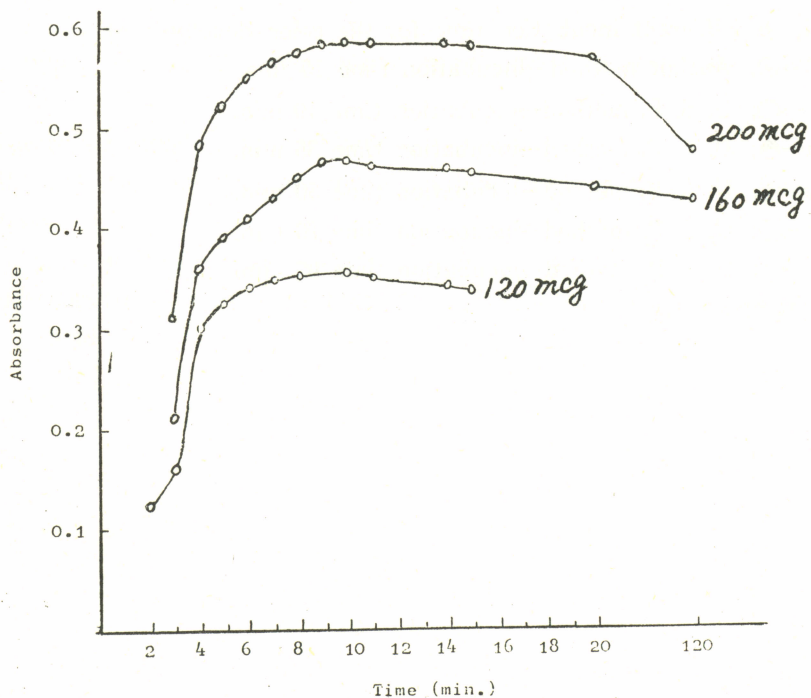


Fig. 8. Rate of colour development—Condition; as in assay procedure without

glacial acetic acid. Read against reagent blank. The result showed that the rate of colour development had decreased slightly after ten minutes, a point it had the maximum absorbance.

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中 文 摘 要

副腎皮質固醇 (Prednisolone) 之吸光度測定 法—氫氧化鉀酒精溶液和冰醋酸之應用

賴 精 二 徐 型 堅

臺北醫學院生化學科 臺北醫學院藥學系

以四重氮苯藍 (Tetrazolium Blue) 作呈色劑，在副腎皮質固醇 (Prednisolone) 之比色定量法中，利用氫氧化鉀酒精溶液代替四甲基氫氧化氮 (Tetramethyl ammonium hydroxide)，在十分鐘內可完成呈色反應，此時加入壹西西冰醋酸或蟻酸來安定反應液。此液可在 $525m\mu$ 之波長下測定其最大吸光度。

四重氮苯藍 (Tetrazolium Blue) 存在下，乙醇本身易發生氧化作用，進而與前者再進行不必要之呈色反應。冰醋酸之加入可阻止乙醇之氧化。又空氣中之二氧化碳易與溶液中之鉀離子作用，此情形不利於定量之操作並影響其準確性。但在冰醋酸之存在下，則可避免此種鉀鹽沉澱的現象。