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# SPECTROPHOTOMETRIC ASSAY OF CERTAIN BENZODIAZEPINE DRUGS THROUGH COMPLEX FORMATION REACTION

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## ABSTRACT

*A simple and accurate spectrophotometric method was developed for the detemination of diazepam, prazepam and flunitrazepam. The method is based on the interaction of the benzodiazepine drug containing an active methylene group with 1,3-dinitrobenzene in presence of tetraethylammonium hydroxide to form a violet coloured σ-complex ( $\lambda_{max}$=560 nm). Beer's law is obeyed over the concentration ranges 2-20 ug ml-1 (for diazepam), 5-30 ug ml-1 (for prazepam) and 5-40 ug ml-1 (for flunitrazepam). The method was successfully applied to the assay of the cited drugs in pure form and in pharmaceutical formulations. Results obtained agree well with those of official methods.*

## INTRODUCTION

The benzodiazepine derivatives are widely used therapeutically because of their relaxant, sedative, hypnotic, anti-psychotic and anticonvulsant properties[1].

Methods to analyze 1,4-benzodiazepine drugs have utilized non-aqueous titrimetry[2,3], UV-spectrophotometry[4,5], visible spectrophotometry[6-9], Fluorimetry[10,11] thin layer chromatography[12,13], gas chromatography[14,15], HPLC[16,17], differential pulse polarography[18,19], radioimmunoassays[20] and radioreceptor assays[21].

Most of the existing colorimetric methods[6-9] involve hydrolysis of the benzodiazepine moiety to benzophenones, and thus lack specificity since this is the usual degradation pathway of these drugs. Therefore, a simple spectrophotometric method for benzodiazepines, involving the intact drug is needed.

All thses requirments are fulfilled in the present investigation by the application of Zimmermann reaction to the active methylene group adjacent to a carbonyl group in some benzodiazepines to produce highly absorbing $\sigma$-complexes upon reaction with 1,3-dinitrobenzene (DNB) in the presence of tetraethylammonium hydroxide (TEAH).

## EXPERIMENTAL

Apparatus :

A Uvidec-320 spectrophotometer (JASCO, Tokyo, Japan) was used.

Materials :

Pharmaceutically pure diazepam, nitrazepam, flunitrazepam, nordazepam, chlordemethyldiazepam, bromazepam, clonazepam and prazepam were obtained from different manufacturers and used as working standards.

The following commercial pharmaceutical preparations were analysed :

Valium ampoules (Roche products, Ltd, UK) each 2 ml ampoule contains 10 mg of diazepam.

Valinil tablets (Nile Co., Egypt), containing 5 mg of diazepam per tablet .

Rohypnol tablets (Roche products, Ltd, UK), containing 2 mg of flunitrazepam per tablet .

Demetrin tablets (Parke Davis, U.S.A.) containing 10 mg of prazepam per tablet.

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Reagents :

1- DNB solution, 1% w/v in ethanol. Prepared from material obtained from BDH, UK.

2- TEAH solution 20% w/v in water, obtained from Riedel-DeHaenag, Germany. Ethanol analytical grade (BDH, U.K.).

Preparation of Sample Solutions :

An accurately weighed amount of the drug (about 25 mg) was dissolved in ethanol and the volume was made up to 25.0 ml with ethanol. This solution was diluted with ethanol to contain 40 $\mu g\ ml^{-1}$ of diazepam or 80 $\mu g\ ml^{-1}$ of flunitrazepam or prazepam.

For injections, an accurately measured volume containing about 25 mg of the benzodiazepine drug, was diluted with ethanaol to contain 40 $\mu g\ ml^{-1}$ of diazepam.

For tablets, 20 tablets were weighed and finely powdered. An accurately weighed portion of the powder, equivalent to about 25 mg of the benzodiazepine drug was extracted twice with 15 ml of ethanol by centrifugation at a speed of 5,000 r.p.m. The supernatent solutions and washings were diluted to 50.0 ml in a volumetric flask with ethanol. This solution was diluted to contain 40 $\mu g\ ml^{-1}$ of diazepam or 80 $\mu g\ ml^{-1}$ of flunitrazepam or prazepam.

General Procedures :

Pipette 1.0 ml of the sample solution of the drug, add 2.0 ml of DNB solution and 1.0 ml of TEAH solution. Mix well and leave for exactly 10 minutes (incases of diazepam and flunitrazepam) or for 20 minutes (in case of prazepam ) at 20 ± 5°C. Record the absorbance of the solutons at 560 nm after the specified time without delay, against a blank similarly treated, substituting 1.0 ml of ethanol for the sample solution. Calculate the concentration of the drug in the sample solution either from properly constructed calibration graphs or from the following linear regression equations:

$$A = 0.0005 + 0.0492\ C \text{ (for diazepam)}$$

$$A = 0.0226 + 0.0272\ C \text{ (for flunitrazepam)}$$

$$A = 0.0001 + 0.0331\ C \text{ (for prazepam)}$$

where A is the recorded absorbance at 560 nm and C is the concentration of the benzodiazepine drug in $\mu g\ ml^{-1}$ in the final assay solution. The correlation coefficients for the above equations are 0.9998, 0.9999 and respectively.

## RESULTS AND DISCUSSION

### Reaction Involved :

The reaction of DNB with active methylene compounds in alkaline medium is known to proceed via the formation of $\sigma$-complexes[22]. The complex is called Meisenheimer complex and the reaction is called the Janovsky reaction. In the presence of excess DNB, the complex is oxidized to a coloured anion while the reagent is reduced to m-nitroaniline under Zimmermann conditions[23], thus the interaction of diazepam with DNB is illustrated by Scheme 1. Kovar and Biegert[24] isolated from the reaction medium of 5 m mole of diazepam and 2.5 m mole of DNB in basic medium a product identified on the bases of $^{1}H$-and $^{13}C$-NMR as a $\sigma$-complex i.e product II (Scheme 1). However, under other analytical conditions i.e. in presence of excess DNB and trace diazepam as it is the case in the proposed assay, the product III (Scheme 1) is more probable, according to the well-known mechanism of Zimmermann reaction[23,25,26].

In the present work, 8 benzodiazepine drugs were tested for colour formation with DNB in the presence of TEAH. The compounds tested were diazepam, nitrazepam, flunitrazepam, nordazepam, chlordemethyldiazepam, bromazepam, clonazepam and prazepam. Among these compouns, only diazepam, flunitrazepam and prazepam gave violet coloured products with $\lambda_{max}$ at 560 nm (Fig. 1).

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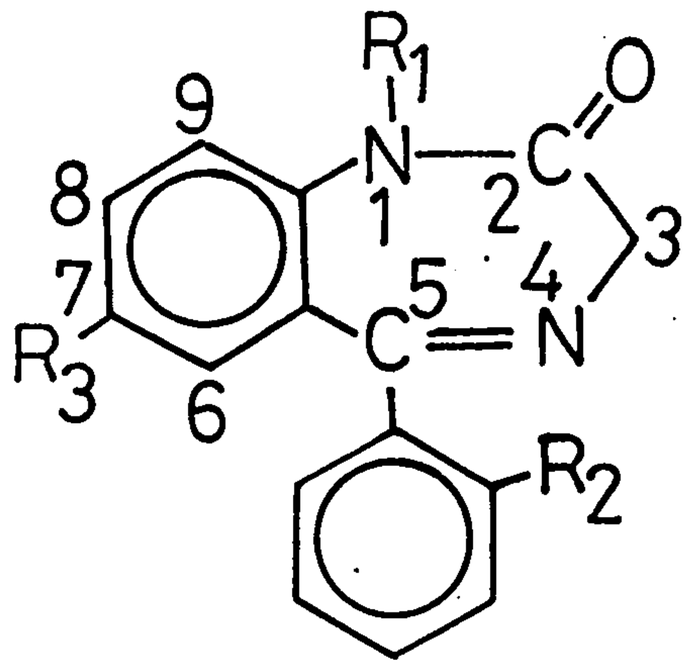

| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Diazepam | $CH_3$ | H | Cl |
| Flunitrazepam | $CH_3$ | F | $No_2$ |
| Prazepam | $CH_2$ | H | Cl |

Based on these findings and the experimental results of Kovar and Biegert with diazepam[24] it can be concluded that :

a) Alkylation of N-1 is an essential structural requirement for the reaction. Although nitrazepam, nordazepam, chlordemethyldiazepam, bromazepam and clonazepam contain an active methylene group adjacent to a carbonyl, none of them yielded $\sigma$-complexes with DNB and TEAH. This may be explained by the (+ I) effect of $R_1$ increasing the activity of the methylene group in the 3-position.

b) An electronegative substituent in C-7 position (e.g. Cl in diazepam and prazepam or $No_2$ in flunitrazepam enhances the reaction[24] .

Optimization of Variables:

The four factors affecting colour intensity are : (a) reagent concentration, (b) type and concentration of base added (c) reaction time and (d) solvent used for preparing drug and reagent solutions. The data presented here illustrate the effect of the

above-mentioned factors on the determination of diazepam as a representative example of benzodiazepine drugs.

Effect of type and Concentration of Polynitro Compound:

Various polynitro aromatics were investigated e.g 1,3,5-trinitrobenzene, 3,5-dinitrobenzoic acid, DNB, picryl chloride,3,5-dinitrobenzonitrile, 1-chloro-2,4-dinitrobenzene and 1-fluoro-2,4-dinitrobenzene. DNB was found to give the highest absorption intensity. When various concentrations of DNB solutions were added to a fixed concentration of the benzodiazepine drug, the absorbances of the resulting solutions were found to increase with increasing reagent concentration up to 10 $\mu g\ ml^{-1}$ in the final assay solution. However, Blank solutions containing higher than 5 $mg\ ml^{-1}$ were relatively dark in colour. The use of 2 ml volume of 1% DNB solution was found satisfactory for the proposed concentration levels of benzodiazepines in this assay .

Effect of Base Type and Concentration :

For the raction between diazepam and DNB, different bases were tried including sodium hydroxide, sodium carbonate basic sodium phosphate, ammonium hydroxide, TEAH, pyridine and N,N-dimethylaniline. Only using sodium hydroxide or TEAH, the characteristic violet colour was formed. The colour obtained with TEAH was 5 times more intense. Optimum concentration of base for maximum intensity of colour was 1 ml of 20% TEAH solution per 4ml of the reaction mixture. Higher concentrations did not significantly increase colour intensity (Fig. 2).

Reaction Time and Stability of Colour :

Maximum colour intensity was obtained within 10 minutes (in cases of diazepam and flunitrazepam) and 20 minutes (in case of prazepam) and stable for further 10 minutes (Fig. 3).

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Effect of Solvent :

The solvent used for dissolving the benzodiazepine drug and DNB largely affect colour intensity of the final solution. Methanol, ethanol, propan-1-ol, propan-2-ol were investigated. Ethanol gave the highest colour intensity (Fig. 4). Some solvents e.g dimethylsulphoxide and acetonitrile interfere in the reaction giving coloured Meisenheimer complexes with DNB and must be avoided. Water causes precipitation of the reagent.

Quantification, Sensitivity, Accuracy and Precision :

A linear correlation was found between absorbance at 560 nm and concentration of the drug in the following ranges; 2-20 $\mu g$ $ml^{-1}$ of diazepam, 5-40 $\mu g$ $ml^{-1}$ of flunitrazepam and 5-30 $\mu g$ $ml^{-1}$ of prazepam. Either linear equations or Beer's plots can be used for calculation of concentration of an unknown sample.

The apparent molar absorptivities of the resulting coloured products are $1.40 \times 10^4$, $8.5 \times 10^3$ and $10.8 \times 10^3$ 1 $mole^{-1}$ $cm^{-1}$ for diazepam, flunitrazepam and prazepam respectively. The minimum detectable amounts were found to be 2 $\mu g$ $ml^{-1}$ of the benzodiazepine derivatives.

To examine the precision of the procedure, six replicate determinations were made on the same solution containing 10 $\mu g$ $ml^{-1}$ of diazepam, 20 $\mu g$ $ml^{-1}$ of flunitrazepam or prazepam. coefficients of variation of 0.43%, 0.77% and 0.98 % respectively were obtained .

Application to Bulk Drugs and Dosage Forms :

Table 1 shows the results obtained for the determination of the investigated benzodiazepines in pure forms and some of their dosage forms. Mean recoveries range from 99.6 to 100.3%. Results obtained with diazepam and prazepam agree well with those obtained by the official methods (Table 1) and thus the proposed method can be recommended for routine utility in drug quality control laboratories being more simple and rapid.

A further advantage of the proposed method is the specificity of the reaction to the intact drug since glycine and o-aminobenzophenone, which are the main degradation products of diazepam, flunitrazepam and prazepam[27], failed to give any colour with DNB and TEAH. Authentic samples of diazepam, when hydrolysed by heating with 0.1 N HCl for 5 minutes on a boiling water bath, failed to gave any detectable colour under the reaction conditions. Standard solutions of diazepam, flunitrazepam and prazepam to which 0.1 mg $ml^{-1}$ of glycine was added when subjected to the proposed procedure gave recoveries ranging from 99.5 to 100.2%. Furthermore, other minor degradation products e.g. carbostyril and acridanone[28] do not contain active methylene group and thus they would not interfere in the proposed method.

Table 1: Determination of some benzodiazepine in bulk materials and in pharmaceutical preparations.

| Sample | DNB method | | | Official method + | t** | F** |
|---|---|---|---|---|---|---|
| | Found* % ± SD | Drug added mg | Recovery* % ±SD | Found* %±SD | | |
| Diazepam | 99.8±0.54 | - | - | 99.6±1.17 | 0.3798 | 4.69 |
| Flunitrazpam | 100.2±0.72 | - | - | - | - | - |
| Prazepam | 99.7±0.89 | - | - | 100.0±0.92 | 0.5735 | 1.07 |
| Valium ampoules | 98.9±0.44 | 10/ml | 100.1±0.62 | 99.1±0.54 | 0.7025 | 1.51 |
| Valinil tablets | 99.5±0.40 | 5/tablet | 99.6±0.58 | 99.8±0.60 | 1.0180 | 2.25 |
| Rohypnol tablets | 99.2±0.78 | 5/tablet | 100.3±0.80 | - | - | - |
| Demetrin tablets | 100.8±0.84 | 5/tablet | 100.0±0.91 | 100.5±0.75 | 0.6519 | 1.25 |

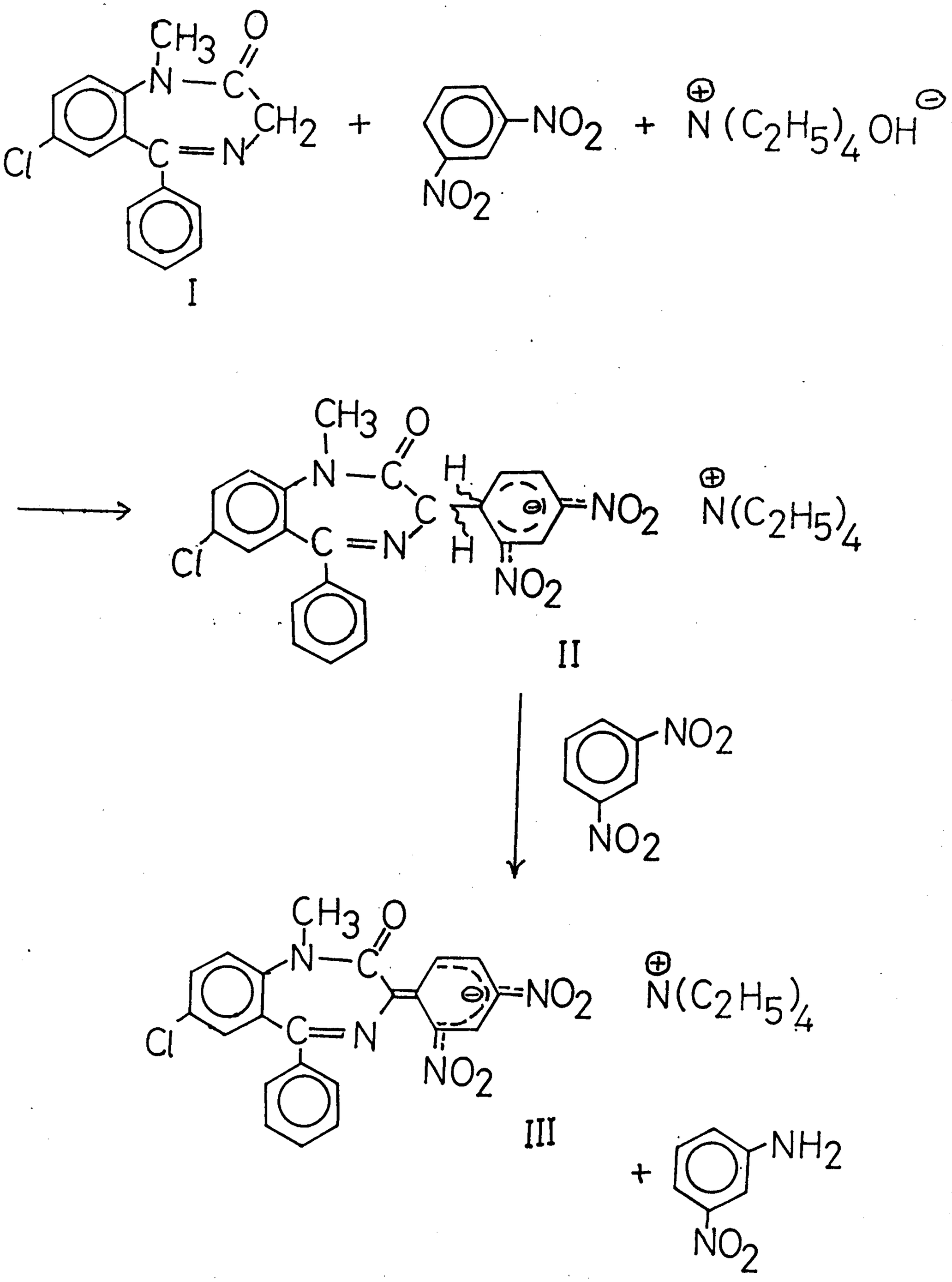
* Average of 6 determinations.

** Tabulated t for 10 degrees of freedom at P 0.05=2.2281

Tabulated F for (5,5) degrees of freedom at P 0.05=5.05

\+ Diazepam was assayed by the B.P. 1980 method while prazepam was assayed by the U.S.P.XXI method.

Flunitrazepam is not official.




Scheme 1: Reaction mechanism

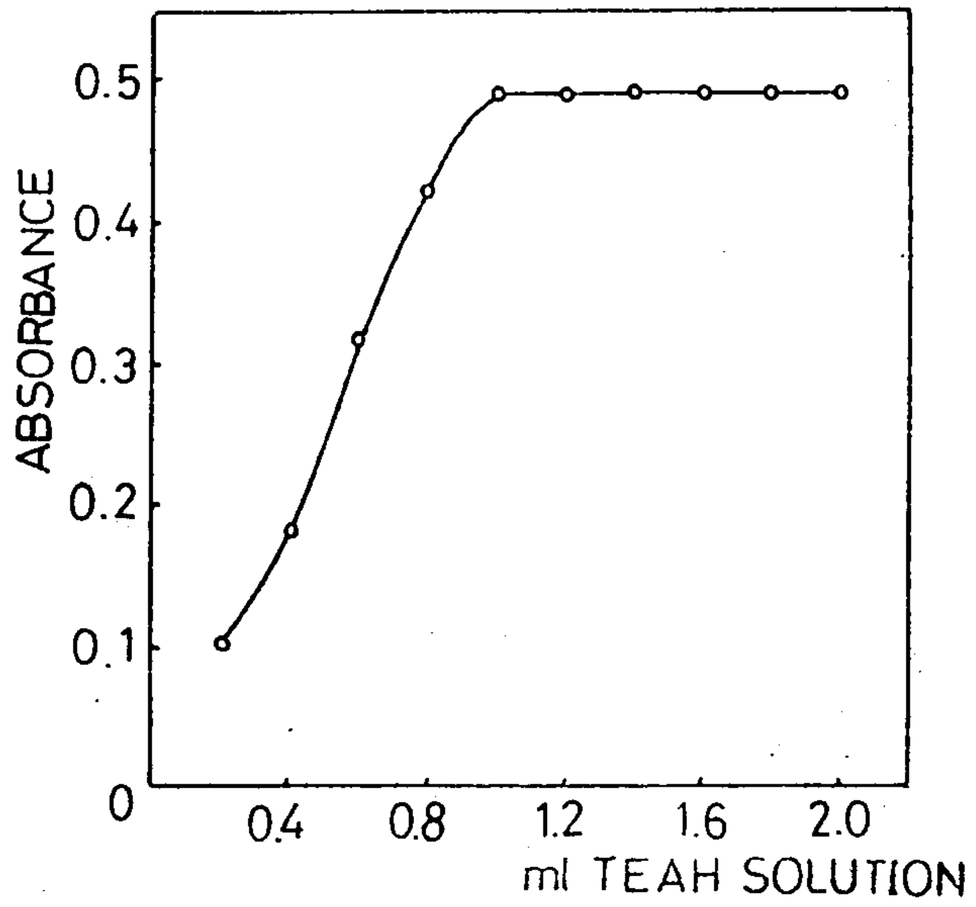



Fig. 2 : Effect of TEAH concentration on the chromogen formed with diazepam.
Final concentration, 10 μg $ml^{-1}$

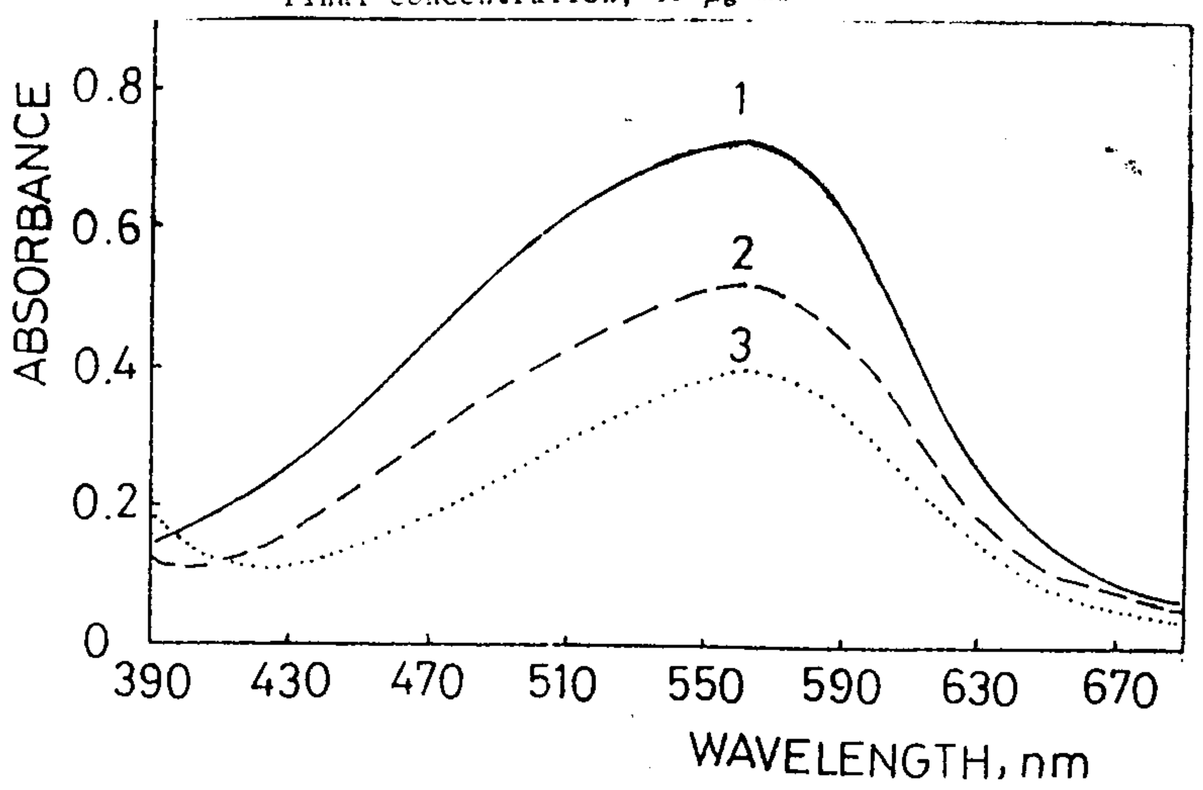



Fig. 1: Absorption spectra of σ-complexes of DNB with: (1) diazepam, (2) parazepam and (3) flunitrazepam.
Final concentration, 15 μg $ml^{-1}$

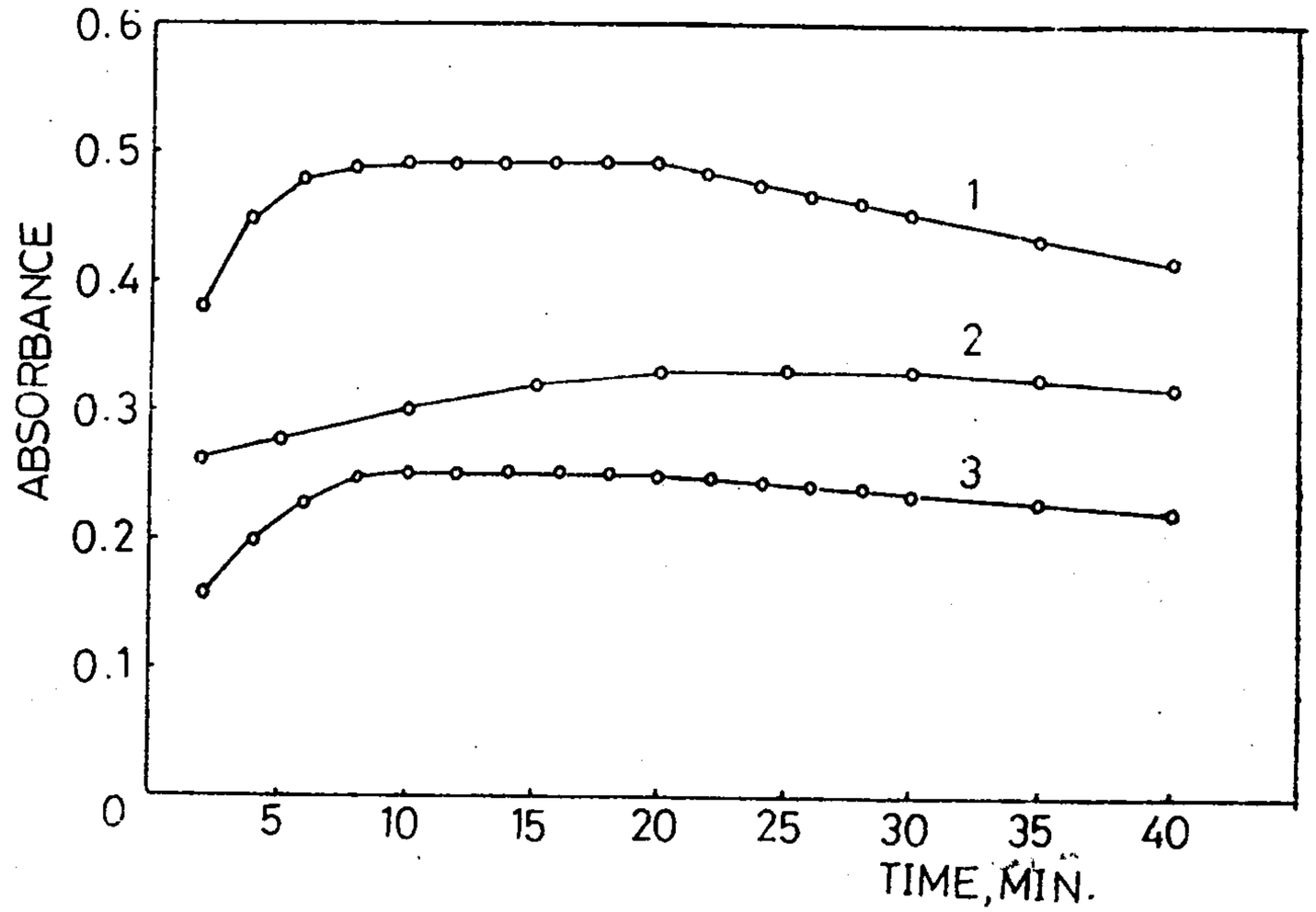



Fig. 3: Colour intensity of benzodiazepine-DNB complex as a function of time.
(1) diazepam, (2) prazepam, (3) flunitrazepam.
Final concentration, 10 μg $ml^{-1}$

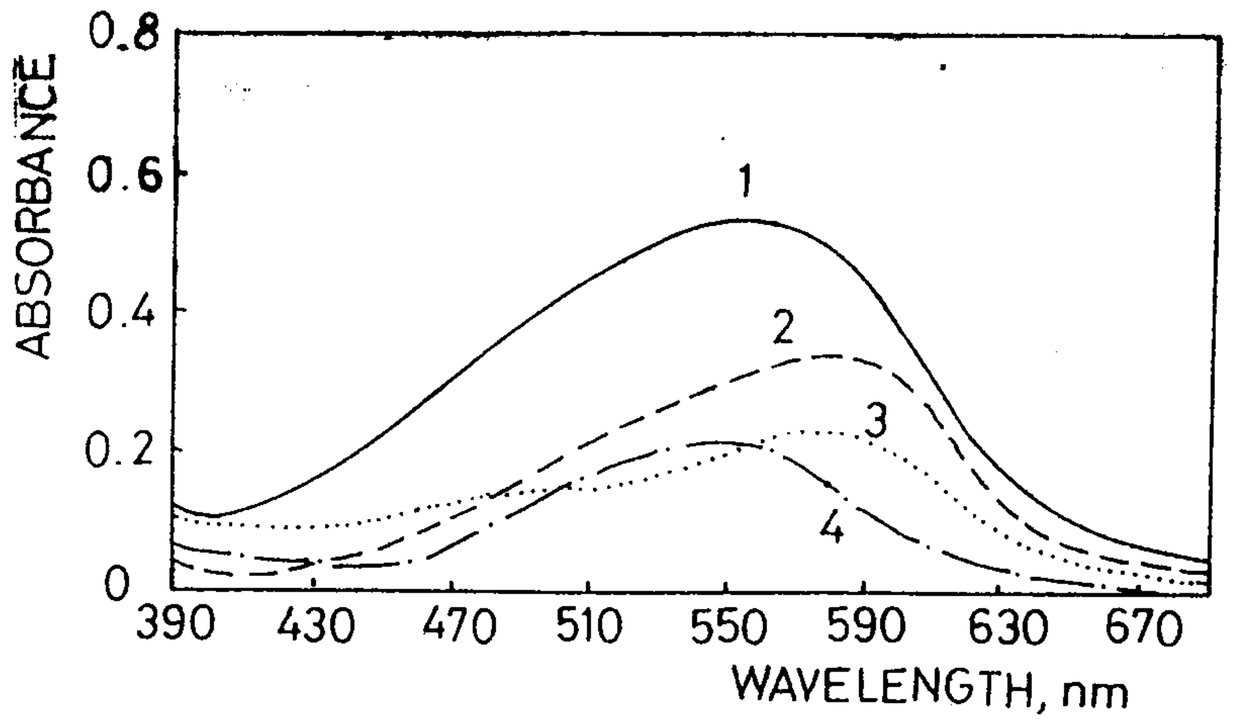



Fig. 4: Effect of solvent on absorption intensity of diazepam-DNB complex.
Final concentration, 10.5 μg $ml^{-1}$

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# تقدير بعض الادوية من مشتقات البنزوديازبين عن طريق تكوين متراكبات

ميشيل ايليا القمص - كاملة محمود عمارة
قسم الكيمياء الصيدلية - كلية الصيدلة - جامعة أسيوط

تم فى هذا البحث التوصل الى طريقة طيفية للتعيين الكمى للديازبام والفلونيترازبام والبرازبام ، وتعتمد الطريقة على تفاعل العقارمع ١ ر ٣ - ثنائى نيتروبنزين فى وجود رباعى ايثيل هيدروكسيد الامونيوم لتكوين متراكب بنفسجى اللون من نوع سيحما له درجة امتصاص عظمى عند موجه طولها ٥٦٠ ن٠م٠

وهذة المركبات تحتوى على مجموعة ميثيلين نشيطة فى الموضع رقم (٣) ومجموعة كربونيل فى الموضع رقم (٢) ومجموعة الكيل متصلة بذرة النيتروجين فى الموضع رقم (١) من نواة البنزوديازين ٠

ويتبع اللون الناتج قانون بيير من ٢ - ٢٠ مكجم / مل للديازبام ، ٥ - ٣٠ مكجم / مل للبرازبام ، د - ٤٠ مكجم / مل للفلونيترازبام ٠

وقد تمت دراسة كل العوامل التى تؤثر على التفاعل وقد وجد أن هذة الطريقة سهلة وسريعة ودقيقة وصالحة للتحليل الروتينى لهذة العقاقير سواء فى المادة الخام أو فى بعض المستحضرات الصيدلية ٠ وقد وجدت النتائج متطابقة مع نتائج الطرق الدستورية ٠

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