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ABSTRACT

Title of Thesis: Detection and Determination of N-nitroso-hexamethyleneimine in Benzenesulfonamide, N-[[(hexahydro-1-H Azepin-1-yl)amino]-Carbonyl]-4 methyl by HPTLC

Anulfo Valdez, Master of Science, 1988

Thesis directed by: Dr. Arthur Greenberg, Professor of Department of Chemical Engineering and Chemistry

A simple method, by High Performance Thin Layer Chromatography (HPTLC), was developed for rapid determination of N-Nitrosohexamethyleneimine in Benzenesulfonamide, N-[[(hexahydro-1 H Azepin-1-yl)amino]-Carbonyl]-4 methyl, (Tolazamide). Separation of N-Nitrosohexamethyleneimine from a Tolazamide solution in chloroform was obtained by precipitation of Tolazamide with n-heptane and by passing the resulting clear solution through a small silica column which was eluted with methanol.

Determination of the N-nitroso compound was achieved by ultraviolet irradiation of it on activated High Performance Silica Gel Plates. Detection was performed by spraying the plates with triethylamine and fluorescamine reagent giving a fluorescent product. Visual detection limits of 25 ppb were observed.

The method is recommended for rapid screening purposes.

DETECTION AND DETERMINATION OF N-NITROSOHEXAMETHYLENEIMINE IN BENZENESULFONAMIDE, N-[[(HEXAHYDRO-1-H AZEPIN-1-YL)AMINO]-CARBONYL]-4 METHYL BY HPTLC

by
Anulfo A. Valdez

Thesis submitted to the Faculty of the Graduate School of the New Jersey Institute of Technology in partial fulfillment of the requiments for the degree of Master of Science
1988

APPROVAL SHEET

Title of Thesis: Detection and Determination of N-Nitrosohexamethyleneimine in Benzenesulfonamide, N-[[(hexahydro-1-H azepin-1-yl)amino]-Carbonyl]-4 methyl By HPTLC

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FOREWORD

The present investigation has been addressed to the analysis of N-nitroso compounds, specifically to the chemical analysis of N-Nitrosohexamethyleneimine in Benzensulfonamide, N-[[(hexahydro-1-H Azepin-1-yl)amino]-Carbonyl]-4 methyl. The last compound, whose generic name is Tolazamide, is used in pharmaceutical industries in the elaboration of drugs destined for use by diabetic patients. The presence of N-nitrosohexamethyleneimine in Tolazamide can be attributed to the synthetic process utilized for its manufacture or to the possibility of a reaction with NO_x in the atmosphere. The importance of sensitive determination of an N-nitrosamine is due to the carcinogenic effects that are well known in this chemical family.

This project presents a new method for detection and determination of N-nitrosohexamethyleneimine impurity in Tolazamide. It can be used as a screening technique for the determination of N-nitroso compounds in bulk materials used in the manufacture of pharmaceuticals, cosmetics, and food products.

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TABLE OF CONTENTS

Chapter page

FOREWORD i

ACKNOWLEDGMENT ii

I. INTRODUCTION 1

1-1. Background 1

1-2. N-nitrosamines 5

1-2-1. Formation and Sources 5

1-3. Tolazamide 8

1-3-1. Structure 8

1-3-2. Reaction of Fluorescamine with 9
1-aminohexamethyleneimine

1-4. High Performance Thin Layer Chromatography 10
(HPTLC)

1-4-1. Characteristics of the Silica Gel Plates.10

1-5. Detection Techniques of N-nitrosamines on Thin..13
Layer Chromatography

II. EXPERIMENTAL SECTION 16

2-1. Apparatus 16

2-2. Materials 16

2-3. Reagents and Solutions 16

2-4. Ultraviolet Light Sources for Irradiation..... 17

2-5. Procedure..... 17

2-6. Visual Detection Limit..... 19

2-7. Safety Precautions..... 19

2-8. Results and Discussion 20

Chapter Page

III. CONCLUSIONS..... 24

APPENDIX A. FIGURES..... 25

REFERENCES..... 36

LIST OF TABLES

Table		page
I.	Survey of Selected Prescription Drugs for..... Possible N-nitroso impurities.	3
II.	Visual Fluorescence Detection Limits of ..... N-nitrosamines on Silica Gel.	11

LIST OF FIGURES

Figure Page

1. High Performance Thin Layer Chromatogram on 26
   silica gel of N-nitrosohexamethyleneimine
2. Ultraviolet Absorption Spectrum of Tolazamide..... 27
3. Ultraviolet Spectrum of the First Derivative of.... 28
   Absorbance Respect to Wavelength of Tolazamide.
4. Ultraviolet Spectrum of the Second Derivative of.... 29
   Absorbance Respect to Wavelength of Tolazamide.
5. Ultraviolet Absorption Spectrum of N-nitroso- 30
   hexamethyleneimine.
6. Ultraviolet Spectrum of the First Derivative of..... 31
   Absorbance Respect to Wavelength of N-nitroso-
   hexamethyleneimine.
7. Ultraviolet Spectrum of the Second Derivative of..... 32
   Absorbance Respect to Wavelength of N-nitroso-
   hexamethyleneimine.
8. Ultraviolet Absorption Spectrum of a Mixture of..... 33
   Tolazamide and N-nitrosohexamethyleneimine.
9. Ultraviolet Spectrum of the First Derivative of 34
   Absorbance Respect to Wavelength of a Mixture
   of Tolazamide and N-nitrosohexamethyleneimine.
10. Ultraviolet Spectrum of the Second Derivative of 35
    Absorbance Respect to Wavelength of a Mixture of
    Tolazamide and N-nitrosohexamethyleneimine.

DETECTION AND DETERMINATION OF N-NITROSOHEXAMETHYLENE-IMINE IN BENZENESULFONAMIDE,N-[[(HEXAHYDRO-1-H AZEPIN-1-YL) AMINO]CARBONYL]-4 METHYL BY HPTLC

CHAPTER I : Introduction

1-1. Background

Since the time carcinogenic effects of N-nitroso compounds were established, many papers have been published on the measurement of the general environmental distribution of such compounds. Fine [14] first associated human cancer with exposure to N-nitroso compounds, whose presence is to be expected wherever, primary, secondary, or tertiary amines are found by reacting with certain oxides of nitrogen [45]. Exposure to N-nitroso compounds or other chemicals can occur by ingestion, inhalation and dermal contact formation. Many pharmaceutical drugs contain chemically bound nitrogen and have sites for possible nitrosation. Therefore, it is extremely important to develope analytical methods capable of detecting part per billion (ppb) levels of N-nitroso compounds, in order to minimize and control human exposure to this clas. Although highly sensitive methods have been developed by using the Thermal Energy Analyzer (TEA) a detector for gas chromatography and high performance liquid chromatography, only a few such methods have been specifically addressed to the analysis of pharmaceutical drugs.

Eisenbrand et al. [12] and Kruel et al. [28] both reported the presence of N-nitroso compounds in several

pharmaceutical products. The former [12], analyzed 68 commercial formulations of the drug aminopyrine and found in all formulations amounts varying from 1 - 370 ug/Kg (ppb) of nitrosodimethylamine. The latter researcher [28] reported that nitrosamine impurities were absent from 68 of 73 pharmaceutical products. Table 1 shows a survey of 32 selected drugs for possible N-nitroso impurities. The method used by this investigator was based on the use of GC-TEA and HPLC-TEA. The TEA detector provides high selectivity to the nitrosyl functional group, allowing detection of mass fractions of less than 10^{-9}. The disadvantages are its price and the special conditions of operation such as the use of traps maintained at very low temperature. In addition, there are other compounds that give false response such as organic and inorganic nitrites, organic nitrates, compounds with labile nitrosyl group and compounds containing certain olefins which chemiluminesce with ozone [16]. In the case of Tolazamide, Kruel et al. [28] screened it from an acetone extract by both GC-TEA and HPTLC-TEA for the presence of N-nitroso compound and found that extraction of Tolazamide in the absence of added sulfamic acid yielded a TEA responsive material by HPLC which co-eluted with an authentic standard of N-nitrosopiperidine. However, when analyzed by gas chromatography, the unknown peak eluted several minutes after N-nitrosopiperidine. Furthermore, when the acetone extract was allowed to stand at room temperature, they observed that the size of the GC-TEA peak increased and when

Table 1

Survey of selected prescription drugs for possible N-nitroso impurities (from reference 28).

ND = none detectable

Chemical definition	Generic name	N-nitroso impurities(ng/g)
1. Phenethylhydrazine sulfate	Phenylzine sulfate	81 ppb
2. 5-(3-Dimethylaminpropyl) -10,11-dihydro-5H-dibenz-[b,f]azepine	Imipramine HCl	68 ppb
3. N-(5-Nitro-2-furfurylidene) -1-aminohydantoin	Nitrofurantoin	40 ppb
4. 3-(o-Methoxyphenoxyl)-1,2-propanediol-1-carbamate	Methocarbamol	ND
5. 4-(5H-Dibenzo[a,d]cyclo-hepten-5-ylidene)-1-methylpiperidine	Cyproheptadiene HCl	ND
6. 2-[p-Chloro-a-(2-dimethylaminoethyl)benzyl]-piperidine	Chlorpheniramine maleate	ND
7. 5-Acetamido-1,3,4-thiadiazole-2-sulfonamide	Acetazolamide	ND
8. Erythromycin	Erythromycin	ND
9. 2-Diphenylmethoxy-N,N-dimethylethylamine	Diphenhydramine	ND
10. Bis(diethylthiocarbamoyl)disulfide	Disulfiram	ND
11. 2-Chloro-5-(1-hydroxy-3-oxo-1-isoindolinyl)-benzenesulfonamide	Chlorthalidone	ND
12. 2-Chloro-10-(3-dimethylaminopropyl)phenothiazine	Chlorpromazine	ND
13. N-(5-Methyl-3-isoxazolyl) sulfanilamide	Sulfamethoxazole	ND
14. 4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydroxyl-6-methyl-1,11-dioxo-2-naphthacene-carboxamide	Tetracycline	ND
15. 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one	Diazepam	ND
16. 2-(p-Bromo-a-(2-dimethylaminoethyl)-benzyl)-pyridine	Parabromdylamine	ND
17. 2-Ethyl-2-phenylglutarimide	Glutethimide	ND
18. 3-Chloro-10-[3-(4-methyl-1-piperazinyl)propyl]-phenothiazine	Prochlorperazine	ND
19. 2-(2,6-Dichloroanilino)-	2-(2,6-Dichloro	ND

Table 1, continued

2-imidazoline	anilino)-2-imidazoline	
20. 2-[Benzyl(2-dimethylaminoethyl)aminopyridine	Tripelennamine	ND
21. 3,3-Diethyl-5-methyl-2,4-piperidinedione	Methyprylon	ND
22. 2-[(2-Dimethylaminoethyl)-(p-methoxybenzyl)amino]-pyrimidine	Thonzylamine-HCl	ND
23. 4-Dimethylamino-3-methyl-1,2-diphenyl-2-butanol-propionate HCl	Propoxyphene	ND
24. 10-[(1-Methyl-3-pyrrolidinyl)methyl]phenothiazine	Methdilazine	ND
25. 10-[3-(Dimethylamino)-2-methylpropyl]phenothiazine	Trimeprazine	ND
26. 1-(Isopropylamino)-3-(1-naphthyloxy)-2-propanol	Propranolol	ND
27. 8-Bromotheophylline-2-amino-2-methyl-1-propanol (1:1)	Pamabrom	ND
28. 2-Sulfanilamidopyrimidine-N-1-1(5-methyl-1,3,4-thiadiazol-2-yl)-sulfanilamide- 2,6-diamino-3-phenylazopyridine HCl	Sulfamethizole-sulfadiazine-phenylazopyridine HCl	ND
29. 1-(Hexahydro-1H-azepin-1-yl)-3-(p-tolylsulfonyl)-urea	Tolazamide	ND
30. 1-Butyl-3-(p-tolylsulfonyl)urea	Tolbutamide	ND
31. [2-(Octahydro-1-azocinyl)ethyl]guanidine	None	ND
32. 7-Chloro-1-[2-(diethylamino)ethyl]-5-(o-fluorophenyl)-3-dihydro-2H, 1,4-benzodiazepin-2-one HCl	Flurazepam HCl	ND

the extraction was repeated in the presence of excess sulfamic acid , little or no GC-TEA responsive material was observed. From these experiments Kruel et al. [28] suggested that the TEA response was not due to N-nitroso derivatives and was due to an interference from the method used in the sample preparation.

1-2. N-nitrosamines

1-2-1. Formation and Sources

For a long time, nitrous acid was used as the main vehicle for nitrosation of amines. Today, it is well known that several other routes are available for the formation of N-nitrosamines. A basic nitrosation reaction in the preparation of N-nitroso compounds which has been studied in detail [13,31], is the reaction between a secondary amine and sodium nitrite under acidic conditions. It has also been demonstrated that primary as well as tertiary amines undergo the nitrosation reaction [18,33]. In the case of tertiary amines although enough evidences have been shown of its reaction with nitrous acid, it is not well accepted in the general literature. Smith et al. [44] proposed that the nitrosation of aliphatic tertiary amines involves electrophilic attack on amine nitrogen followed by 1,2 elimination to give an imminium compound which is hydrolyzed to the final products. Ohshima et al.[33] reported kinetic data on the nitrosation of Me_3N and Me_3NO at 25-100° and pH 1-7 to give Me_2NNO(1) and suggested that the formation of

(1) at higher temperatures involves the oxidative cleavage of the tertiary amine to produce Me_2NH, which reacts with nitrite to form (1). At lower temperatures they suggested that (1) is formed from Me_3N and Me_3NO through a pathway not forming Me_2NH.

Another route for the formation of N-nitrosamines involves a reaction called transnitrosation, in which the nitrosyl group of an N-nitrosamine is transferred to a secondary amine. Transnitrosation can also occur from aromatic N-nitroso derivatives as well as from other nitrosyl donors such as C-nitroso and S-nitroso compounds [2, 4, 6, 43].

N-nitroso compounds can also be expected in any amine-containing formulation which has been stored or transported in nitrite - treated metal containers [31]. DuPont de Nemours & Co., Inc. showed that, in Benzac, the nitrosodimethylamine level was reduced from 180 ug/ml to 2 ug/ml by changing the final commercial package from a nitrite treated metal can to a plastic-lined container [3]. In general, many synthetic routes involve the use of a nitrosating agent and if an amine is subsequently added to the process or if the final product is an amine, then residual nitrosating agent may have formed N-nitroso compounds. This mechanism should apply to all synthetic compounds, including food dyes, industrial chemicals and pharmaceuticals. A good example of this pathway is the synthetic route for the manufacture of a emulsified

concentrate of alpha, alpha, alpha-trifluoro-2, 6-dinitro-N, N-dipropyl-p-toluidine (Treflan) [38].

High concentrations of nitrosamines have been reported in the workplace [9]. The rubber industry uses nitrosodiphenylamine as a vulcanization retarder. This compound is labile and can participate in transnitrosation reactions, given other carcinogenic N-nitroso compounds. Concentrations of Nitrosodiphenylamine varying from 0.2 to 47 ug/m³ have been reported in the air of rubber factories [9]. Levels of Nitrosomorpholine ranging from 0.5 - 27 Ug/m³ were reported in curing and extrusion sections of the rubber factories [9]. Levels of Nitrosomorpholine and nitrosodimethylamine in the rubber industry have been reported by Mcglothlin et al. [30] and by Preussmann et al. [35]. Chemical plants that use nitrosodimethylamine and chemical plants manufacturing dimethylamine have been considered as main sources of the presence of nitrosodimethylamine in the enviorenment [15, 17].

Nonoccupational exposure to N-nitroso compounds have been attributed to tobacco smoke, and is greater than all other nonoccupational exposures combined: food, meats, dairy products, fish, alcoholic beverages, cosmetics, pesticides, water, air and pharmaceuticals [8, 10, 19, 31, 39]. In cosmetics, as well as in pharmaceuticals, the precise source of the nitrosating agent is not yet known. However, several possibilities exist. First, many cosmetics contain

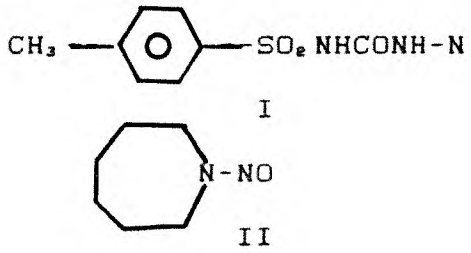
formaldehyde releasing compounds which are used as preservatives; formaldehyde is known to catalyze N-nitrosation reactions under certain conditions [10]. Second, many cosmetics contain C-nitro bactericides which may be involved in transnitrosation. Third, the chemicals used in cosmetics are crude and may contain a myriad of other products, some of which may be nitrosating agents. Fourth, some of the ingredients used in comestics may be shipped or stored in metal containers which have been treated with nitrite.

Many prescription and non-prescription drugs contain secondary and or tertiary amine type structures and N-nitroso compounds can be produced by reaction with nitric oxides (NO, NO_2) in the air, and by the synthetic process used in its manufacture.

1-3. Tolazamide

1-3-1. Structure

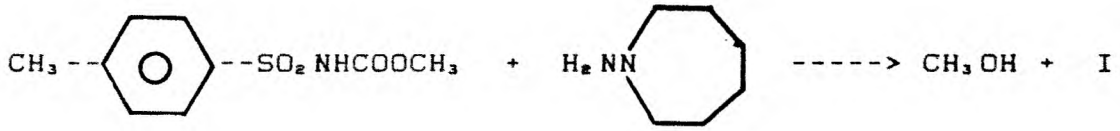
The chemical structures of Tolazamide (I) and its N-nitroso impurity N-nitrosohexamethyleneimine (II) are shown below.



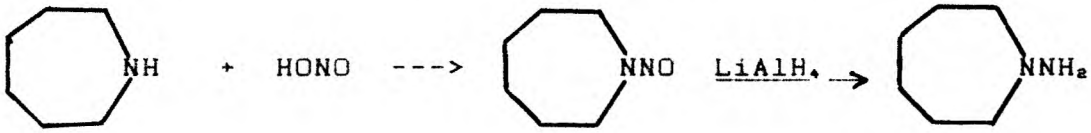
I

II

Tolazamide can be prepared by treatment of its arylsulfonylurethanes with its corresponding hydrazines, according to the general method of Marshall and Sigal [29].

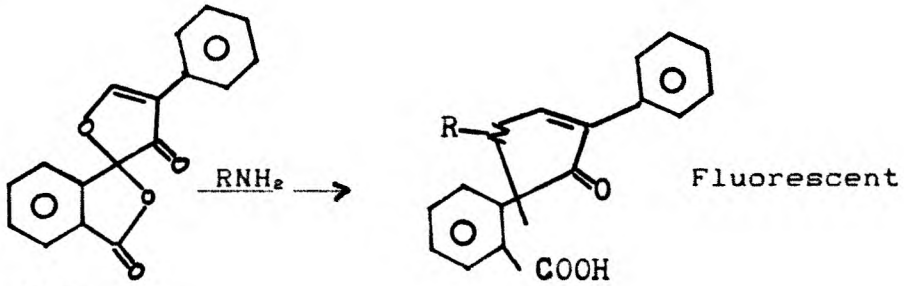


The requisite hydrazine needed is prepared by treatment of the corresponding nitrosamine with lithium aluminum hydride.



1-3-2. Reaction of Fluorescamine with 1-aminohexamethyleneimine

Fluorescamine is the common name for 4-phenylspiro[furan-2(3H),1'phthalan]-3,3'-dione (III). This reagent reacts directly with primary aliphatic or aromatic amines to form a fluorophor of high fluorescence as shown below.



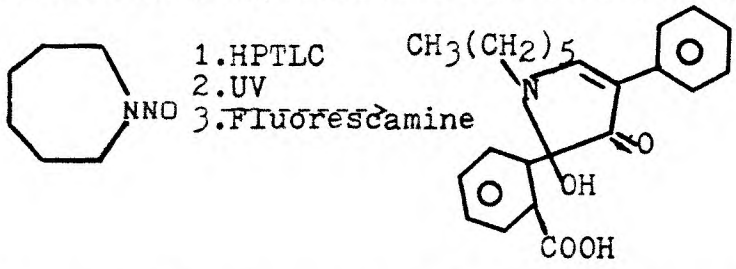
III

The fluorophors obtained by this reaction exhibit high fluorescence yields and are thus very sensitive probes and

have similar excitation-emission spectral characteristics [7,23].

Young [48,49] has described a detection method for N-nitrosamines on thin layer chromatographic silica gel plates based on ultraviolet irradiation. He studied, the 24 nitrosamines listed in Table 2, and found detection limits between 8 and 500 ng. The N-nitrosamines yield the corresponding primary or secondary amines by cleavage with ultraviolet light on activated silica gel plates. Our investigation was carried out by using high performance thin layer chromatographic silica gel plates whose characteristics will be described later.

The reaction process for the detection of N-nitrosohexamethyleneimine can be shown as follow:



1.4. High Performance Thin Layer Chromatography (HPTLC)

1.4.1. Characteristics of the silica gel plates

High Performance Thin Layer Chromatography is characterized by use of glass plates precoated with a silica gel layer consisting of an extremely dense packing of small particles of very uniform size, 4.5 u, with a smooth, homogeneous surface. The use of small particles on HPTLC allow very good contact to be made between the sample, the

Table 2
Visual fluorescence detection limits of N-nitrosamines on silica gel (from reference 49).

N-nitrosamine	Detection limit, ng
1. Dimethyl	500
2. Diethyl	10-40
3. Dipropyl	7-10
4. Dibutyl	9-12
5. Dipentyl	4-6
6. Dihexyl	9-12
7. Diheptyl	9-12
8. Dioctyl	8-11
9. Diallyl	10-12
10. Di-iso-butyl	8-11
11. Dicyclohexyl	12-14
12. Dibenzyl	15-18
13. Methyl, butyl	7-10
14. Ethyl, butyl	8-11
15. Methyl, phenyl	17-20
16. Propyl, phenyl	10-12
17. Ethyl, benzyl	16-20
18. Phenyl, benzyl	7-10
19. Pyrrolidinyl	20-40
20. Piperidinyl	17-20
21. Diphenyl	nf
22. Morpholinyl	nf
23. N-Methyl piperazinyl	nf
24. Carbazolyl	nf

nf = non-fluorescent products.

mobile phase and the adsorbent surface. The small adsorbent particles improve efficiency to such an extent that the separation can be performed in 3 to 6 cm of running distance compared to 15 to 20 cm in conventional thin layer chromatography(TLC). This means that the separation is completed in 1/5 or 1/10 of the time needed for conventional TLC and less mobile phase is required.

The major advantage of HPTLC as compared to other chromatographic methods is the speed of analysis on a per sample basis. This is due to the ability to spot multiple samples on a single plate, the short development distance and resultant short development time. Spotting samples along with standards on the same plate allows them to be processed under identical conditions (in contrast to sequential analysis on a column) for maximum quantitative accuracy and reproducibility.

Time is also conserved by optimizing the separation conditions for the compounds of interest, while others can be left at the origin or in the poor resolution, high R_f region of the plate. The R_f is the ratio of the distance moved by spot and distance moved by solvent front [22]. However, complex mixtures can also be effectively resolved by using multiple or two-dimensional development with a variety of mobile phases. Solvent systems for the two-dimensional separation of amino acids that are suitable for HPTLC are described by Seiler and Knodgen [41]. The choice of mobile phase components is not restricted by concerns

about deterioration of the silica gel, since layers are not reused.

These characteristics have made possible the application of HPTLC to the analysis of amino acids and proteins [1,20,41], carbohydrates [24,32], antibiotics [25,26,34], drugs and pharmaceuticals [5,11,21,27,40].

Whatman HPTLC plates, LHP-K, were selected for the realization of this project. This type of plate has a preadsorbent area which represent is advantageous. The preadsorbent area allows the application of volumes many time larger than those normally recommended for HPTLC. The Whatman preadsorbent area consists of a 2 cm strip of inert material that acts as an inactive blotter or staging area for application of samples. The composition of this inert material is characterized by high purity, high specific surface area, low overall density, extremely high porosity, insolubility, high fusion point, chemical inertness, and low reactivity at normal temperatures. Samples can be applied rapidly to the preadsorbent and then dried thoroughly, and there is little chance of the decomposition that can sometimes occur when applying spots to active adsorbent surfaces like silica gel or alumina. Other characteristics and properties of HPTLC plates are described by Halminton et al. [22].

1.5. Detection techniques of N-nitrosamines on thin layer chromatography.

A number of detection techniques have been developed for detecting N-nitrosamines. Among the usual TLC procedures is the use of Preussmann reagent. This reagent consists of 5 parts of a solution of diphenylamine,(1.5% in ethanol) and 1 part of a solution of palladium chloride (0.1% in 0.2% saline). Detection is based on the irradiation, for several minutes, of the sprayed plate with ultra-violet light; blue to violet spots of fluorophors derived from nitroso compounds are produced and the detection limits are usually 0.5-2 ug [36]. The mechanism of the reaction is based on a photochemical transnitrosation reaction with transfer of the nitroso group to diphenylamine, the color of the formed p-nitroso-diphenylamine is produced by its reaction with palladium chloride [47]. Yang and Brown et. al. [46] have reduced a number of nitrosamines to hydrazines, with lithium aluminum hydride in tetrahydrofuran, and condensed with 9-anthraldehyde and 9-phenanthraldehyde. The resulting hydrazones are separated by thin layer chromatography and detected by exposing the plate to ultraviolet light or iodine vapor. Sen et. al.[42] has used for the detection of nitrosamines, ultraviolet irradiation and treatment with ninhydrin and has reported a detection limit as low as 50 ng (for N-nitrosopyrrolidine.)

The detection procedure developed by Young, as it was described before, seems to be extremely sensitive for the detection of N-nitrosamines on thin layer chromatography.

Finally, the technological advances on densitometric instruments have provided quantitation in HPTLC and in TLC by photometric measurements of the absorption of colored or quenched spots, or by measurements of emitted fluorescence. A good description of different commercial instruments is given by Prosek [37].

The goals of this project were: first, to develop an analytical method which can be used for rapid screening purposes in the manufacture of the pharmaceutical drug, Tolazamide; second, to develop an analytical technique that responds to the aceptable level established by the Federal Drug Administration (FDA),of 100 ppb; and third, to set parameters and procedures for the analysis of N-nitrosohexamethyleneimine in tolazamide.

CHAPTER II: Experimental Section

2-1. Apparatus

The ultraviolet absorption spetrums were taken with a Hewlett Packard Spectrophotometer model 8450A. The HPTLC plates were developed in a 27 x 9 1/2 x 29 cm glass chamber.

2-2. Materials

All the solvents and reagents were of analytical grade. Fluorescamine (Fluram) was obtained from Roche Diagnostics, Division of Hoffmann-La Roche Inc., NJ; Ninhydrin and Palladium chloride were purchased from J.T. Baker Chemical Co., and Diphenylamine from Mallinckrodt Inc. The standards, Tolazamide and N-nitrosohexamethyleneimine were supplied by Zenith Laboratories, Northvale, NJ. The HPTLC silica gel plates, LHP-K 10 x 10cm, were purchased from Whatman Co., and adsorbent silica gel, 70-150 mesh, from Universal Scientific Inc.

2-3. Reagents and solutions

Solutions of 0.1 mg/ml fluorescamine in acetone were freshly prepared each time prior to use. Solutions of 10% triethylamine in methylene chloride were prepared each three days. Both reagents were stored at room temperature in a stoppered vessel. The stock solution, 0.5 mg/ml, of N-nitrosohexamethyleneimine was prepared in chloroform and stored in the refrigerator for period of time no longer than

a week. Prior to use it several aliquots were taken to prepared a final solution of 50 ng/ml.

2-4. Ultraviolet light sources for irradiation

Two combined ultraviolet (UV) light sources were used: a ultraviolet fluorescence analysis cabinet; Spectroline model CX-20, and an auxiliary portable UV lamp Spectroline model 0-22SNF, 118 volt, 60 Hz, 130 amps. Both sources were examined for their ability to cleave the N-nitrosohexamethyleneimine photolytically on high performance thin layer chromatography plates.

2-5. Procedure

The experimental procedure was based on the preparation of spiked Tolazamide standards, having a concentration of 50 ppb of N-nitrosohexamethyleneimine. One gram of the bulk material, Tolazamide, was weighed out in a 25ml centrifuge tube and dissolved in 6 ml of chloroform. Ten ml of n-hexane was then added, mixed and centrifuged for 20 minutes at about 2500 rpm. The volume was evaporated to about 6 ml by a current of nitrogen on a Pierce Reacti, Heating module and temperature of 50°C. In this step most of the Tolazamide is separated. The solution of about 6 ml wich contents the N-nitroso impurity was transferred to a 2.5g silica gel packed column and eluted with 3ml of methanol. The eluted solvent was collected into a 10ml conical centrifuge tube and evaporated to almost to dryness under a current of nitrogen and low temperature; stoppered and stored until spotting on the HPTLC plate.

The HPTLC plate was predeveloped prior to used in a mixture of hexane, ether and dichloromethane (4:3:2), and activated at 110 °C for one hour. The HPTLC plate was predeveloped and in a glass chamber having the following dimension: 27cm high, 29cm long and 9 1/2 cm wide. The remaining volume of about 50 ul containing the N-nitrosohexamethyleneimine was spotted in the preadsorbent layer of the plate with a Hamilton microsyringe of 100 ul. The total volume was spotted in several small amounts each of them followed by drying. In addition to the spiked sample, standards of N-nitrosohexamethyleneimine of 25, 50 and 100 ng were spotted. The plate was developed 5.5 cm in a saturated chamber of the mixture of solvents mentioned above.

At first, the developed plate was visualized by three different ways. First, the developed plate was sprayed with diluted aqueous acetic acid, irradiated with UV light for 10 minutes and sprayed with a 0.3% Ninhydrin solution and 2% pyridine in ethanol and heated at 80 °C for 20 minutes. The expected red purple spots were not present although is well known that Ninhydrin reacts with primary and secondary amines. Second, the developed HPTLC plate was sprayed with Preussmann reagent and irradiated for 10 to 60 minutes with UV light. Again, the expected purple spot was not present. It was found that Preussmann reagent gave positive results at level above 500ng of N-nitrosohexamethyleneimine and after long exposure to UV light. Finally, the plate was

irradiated for a period of 10 minutes and treated by the following fluorescamine spray procedure:

1) Spray with a solution of 10 % triethylamine in methylene chloride; air dry for several seconds.

2) Spray with a solution of 0.1 mg/ml fluorescamine in acetone; air dry for several seconds.

3) Re-spray with a solution of 10% triethylamine in methylene chloride.

4) Re-exposure to UV light for 10 minutes.

The plate was viewed in a fluorescence cabinet under short (254nm) wavelength ultraviolet light. Fluorescent spots were obtained for all amount spotted at Rf value of 0.65.

2-6. Visual detection limit

The detection limit for the visual fluorescence of N-nitrosohexamethyleneimine was determinated by spotting decreasing volumes of standard solution of the N-nitroso impurity on activated Whatman HPTLC silica plate, developing, irradiating, spraying with fluorescamine reagent, and viewing under UV light.

2-7. Safety Precautions

Extremely safety precautions were taken at all moments to prevent skin contact and inhalation of the N-nitroso compound investigated, due to its carcinogenic effects. In addition precautions were also taken when viewing the plates in the ultraviolet fluorescence cabinet to avoid direct exposure of the eyes with the UV light.

2-8. Results and Discussion

For the first time, evidences have been shown for the formation of a primary amine from N-nitrosohexamethyleneimine. Since only primary amines give fluorescent pyrrolidonenes by reaction with fluorescamine. A sucessful analytical technique was developed for the determination of N-nitrosohexamethyleneimine in bulk material, Tolazamide. As little as 25 ng of N-nitrosohexamethyleneimine were visually detectable. A semiquantitative result was obtained by comparing the intensity of the sample spot with those of the standards at R_f value of 0.65 as shown in figure 1. Since the concentration of N-nitrosohexamethyleneimine in the spiked sample was of 50 ng/g (ppb), the standards amount spotted were of 25, 50 and 100 ng. In this way a semiquantitative estimation of N-nitrosohexamethyleneimine was obtained.

In the separation process, the use of heptane allowed a preliminary separation of the Tolazamide from the N-nitroso impurity, which is soluble in chloroform and heptane; while the Tolazamide is practically insoluble in heptane. In the course of this investigation heptane-extractions of Tolazamide were done and although good results were obtained, the extraction-time required was of several hours increasing this, the risk of losing the sample by the effect of prolonged shacking. The complete dissolution of Tolazamide in chloroform was selected rather than the "heptane-wash" of it. A complete separation of N-nitroso-

hexamethyleneimine from Tolazamide was achieved by passing the final solution through the silica gel column. Different mesh size and brands of silica gel were used; Whatman, Merck and Woelm Pharma. The latter, from Universal Scientific Inc., was chosen since was packed properly; aluminum bottle, and its purity was superior to the other brands mentioned above.

The determination of the eluting solvent and volume was obtained by passing standards containing Tolazamide and N-nitrosohexamethyleneimine through a silica gel column. First of all, ultraviolet absorption spectrums in the range 200 - 400 nm were taken in a 1 cm cell. Spectrums of Tolazamide, N-nitrosohexamethyleneimine and mixture of both were taken to establish its differences. Figure 2 shows the ultraviolet absorption spectrum of Tolazamide having a very sharp peak with maximum at about 276 nm. The ultraviolet absorption spectrum of N-nitrosohexamethyleneimine is shown in figure 5 having maximum at about 356 nm. The ultraviolet absorption spectrum of both, Tolazamide and N-nitrosohexamethyleneimine is presented in figure 8 showing a well defined peak with maximum at about 356 nm (N-nitrosohexamethyleneimine) and a less defined peak at about 276 nm (Tolazamide). Since the presence of Tolazamide was poorly observed in the mixture as seen from figure 8, first (da/dl) and second (d^2a/d^2l) derivative of absorbance respect to wavelength of Tolazamide, N-nitrosohexamethyleneimine and of a mixture of both were taken and are shown in figure 3, 4,

6, 7, 9 and 10. Figure 10, spectrum of the second derivative of absorbance respect to wavelength of a mixture of both compounds, presents three well defined peaks in the range 260-285 nm corresponding to Tolazamide whose similar spectrum is shown in figure 4. The use of these spectra to screen the presence of Tolazamide allowed the selection of the eluting solvent. The column was eluted with several organic solvents such as hexane, methanol and chloroform and was monitored spectrophotometrically as it was already described. Either methanol or chloroform can be used as eluting solvent. Methanol was selected for being less harmful than chloroform.

The irradiation time with ultraviolet light was determined by measuring the time-exposure as well as the irradiation-distance. The irradiation-time of 2 periods of 10 minutes each as was already described showed the best results, since longer exposure to UV-light do not improve the fluorescence of the spots. The fluorescence cabinet used for the irradiation process fixed the irradiation distance which was of 8 cm. It was also found that the fluorescence of the spots vanish after a period of about 12 hours.

Although the volume of sample applied to the preadsorbent layer of the HPTLC plate was between 50 to 100ul, the efficiency was found to be uniform due to the fact that the total volume was spotted in small amounts each of them followed by drying. In addition, if any small amount of Tolazamide is spotted , it will be retained in the

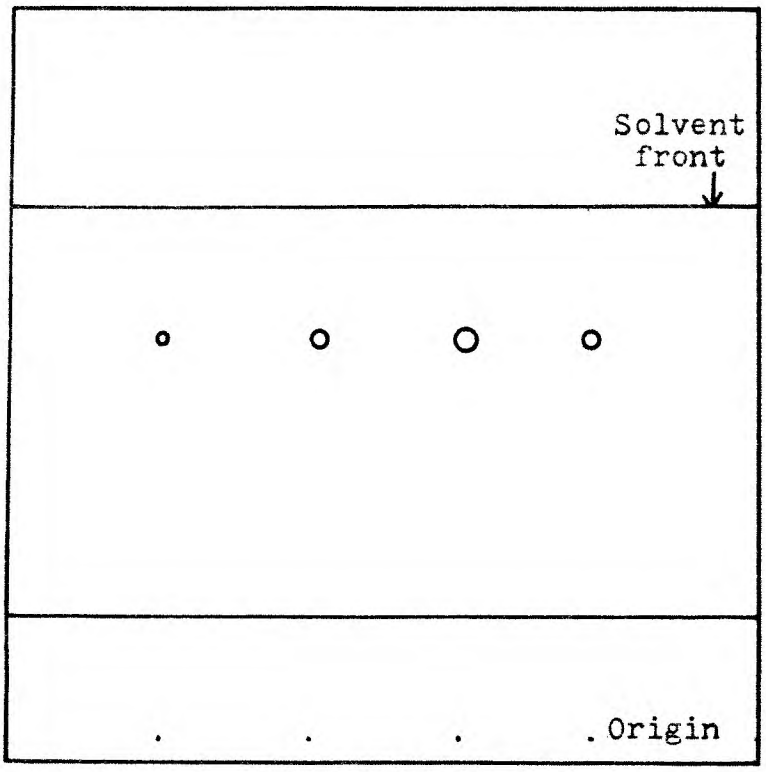
preadsorbent layer or very down in the plate since its mobility is restrained by its chemical structure and high molecular weight.

CHAPTER III. Conclusion

High Performance Thin Layer Chromatography was used to determine the presence of and provide a semiquantitative value for N-nitrosohexamethyleneimine in Tolazamide. The semi-quantitative results obtained by the method of analysis developed in the present work were excellent since the intensity of the spot obtained from the spiked sample containing 50ppb was visually the same to the intensity of the spot corresponding to the 50ng standard of N-nitrosohexamethyleneimine. Further investigation can assure the utility of HPTLC in the rapid determination of N-nitroso compounds in other pharmaceutical products, using as a basis the present thesis.

The method developed is being successfully used as a screening technique to detect the presence of N-nitrosohexamethyleneimine in Tolazamide whose limit should be not more than 100 ppb. It is my opinion that quantitative methods can be achieved by using HPTLC with densitometric scanning. Such methods would offer the following advantages as compared to other chromatographic methods such as HPLC and GC; ability to spot multiple samples on a single plate, short development distance and resultant short development time. On the other hand, spotting samples along with standards on the same plate allows them to be processed under identical conditions for maximum quantitative accuracy and reproducibility.

APPENDIX A: FIGURES



o=Standard 25 ppb O=Standard 50ppb

O=Standard 100 ppb =Spiked sample 50 ppb

Figure 1. High Performance Thin Layer Chromatogram on silica gel of N-nitrosohexamethyleneimine

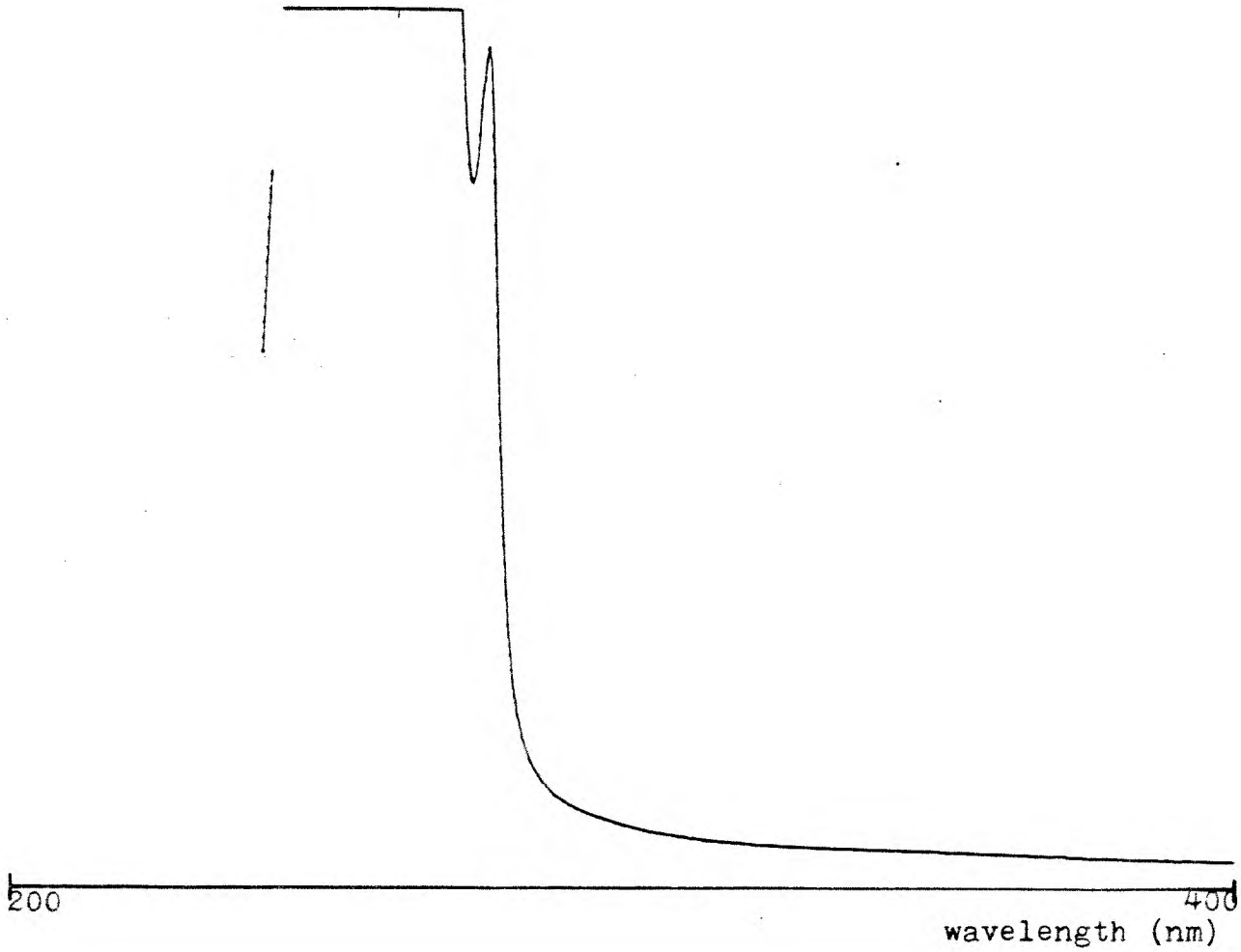


Figure 2. Ultraviolet Absorption Spectrum of Tolazamide

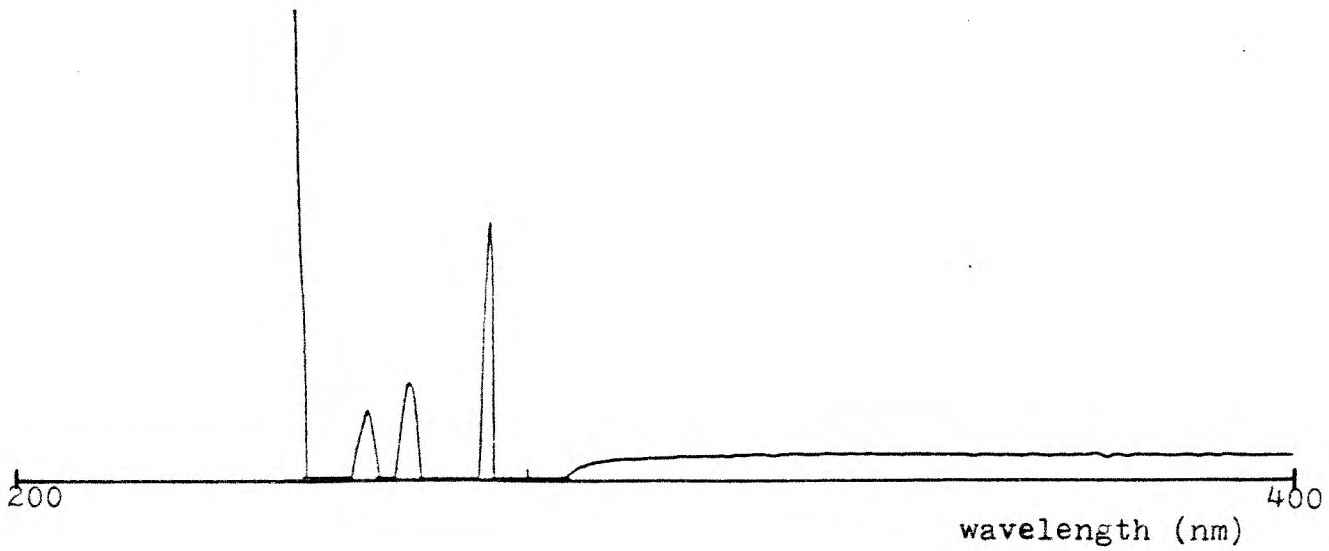


Figure 3. Ultraviolet Spectrum of the First Derivative of Absorbance Respect to Wavelength of Tolazamide

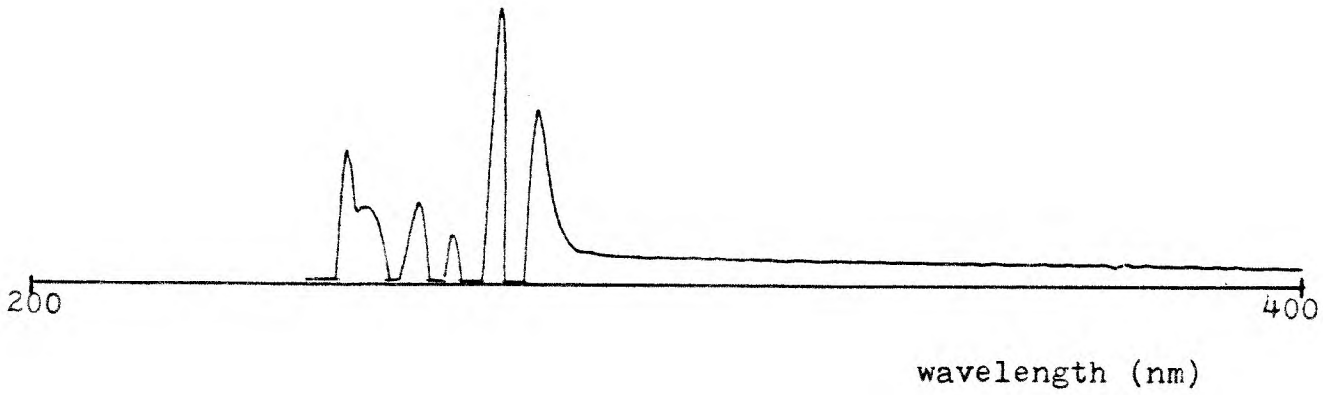


Figure 4. Ultraviolet Spectrum of the Second Derivative of Absorbance Respect to Wavelength of Tolazamide

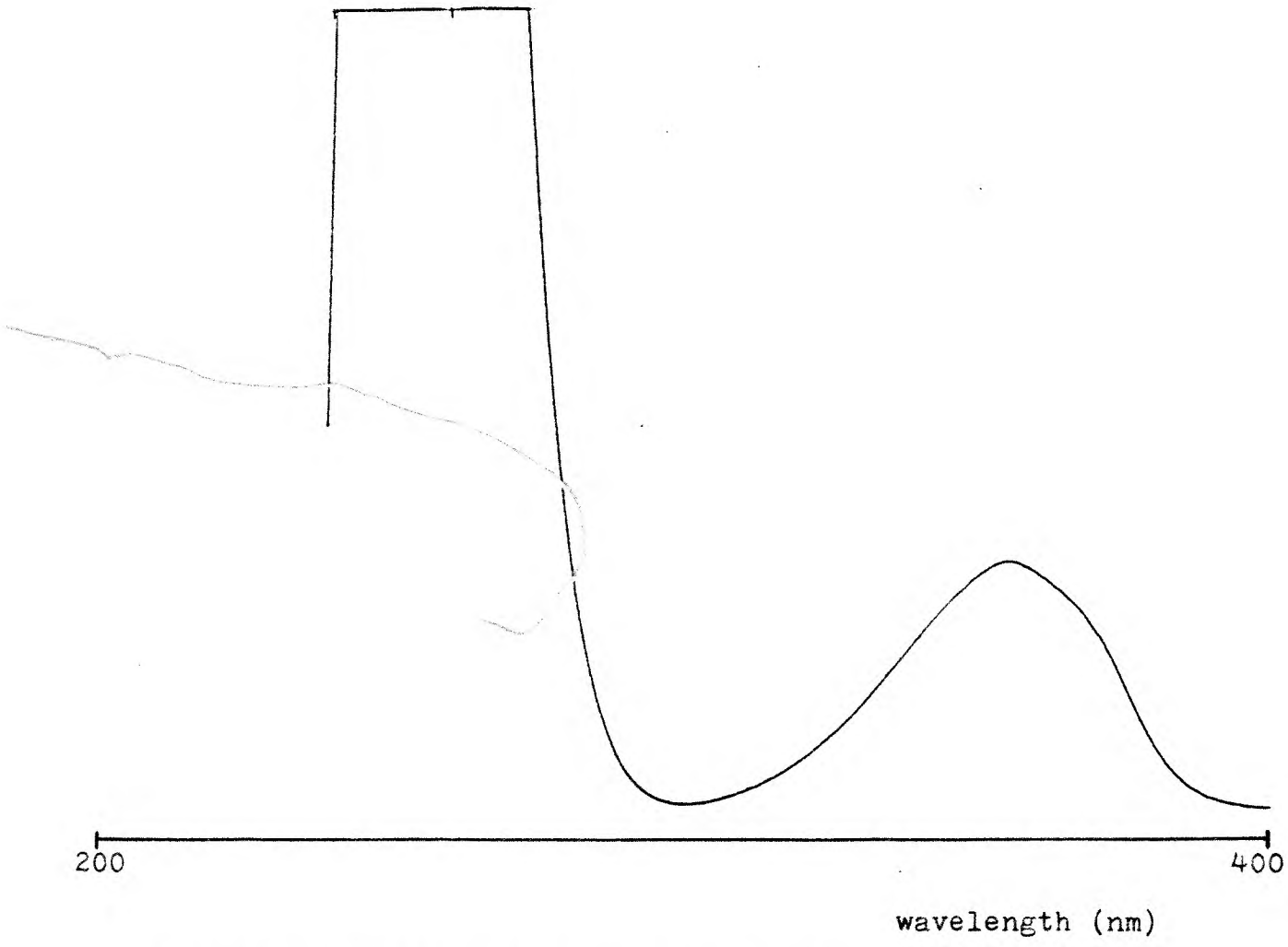


Figure 5. Ultraviolet Absorption Spectrum of N-nitroso-hexamethyleneimine

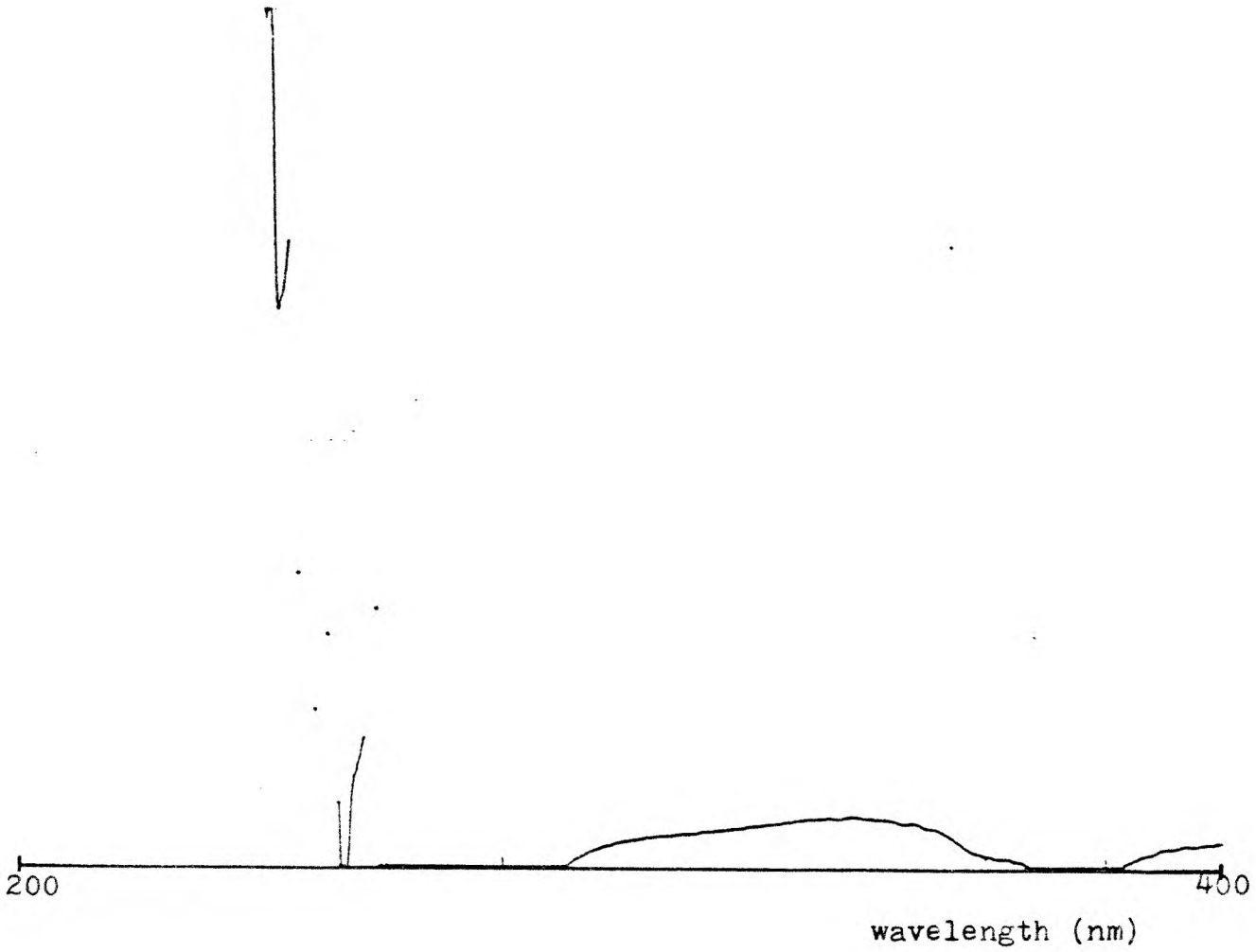


Figure 6. Ultraviolet Spectrum of the First Derivative of Absorbance Respect to Wavelength of N-nitroso-hexamethyleneimine

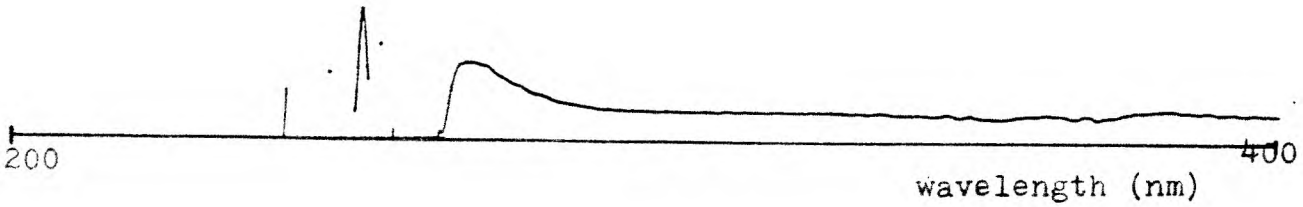


Figure 7. Ultraviolet Spectrum of the Second Derivative of Absorbance Respect to wavelength of N-nitrosohexamethyleneimine

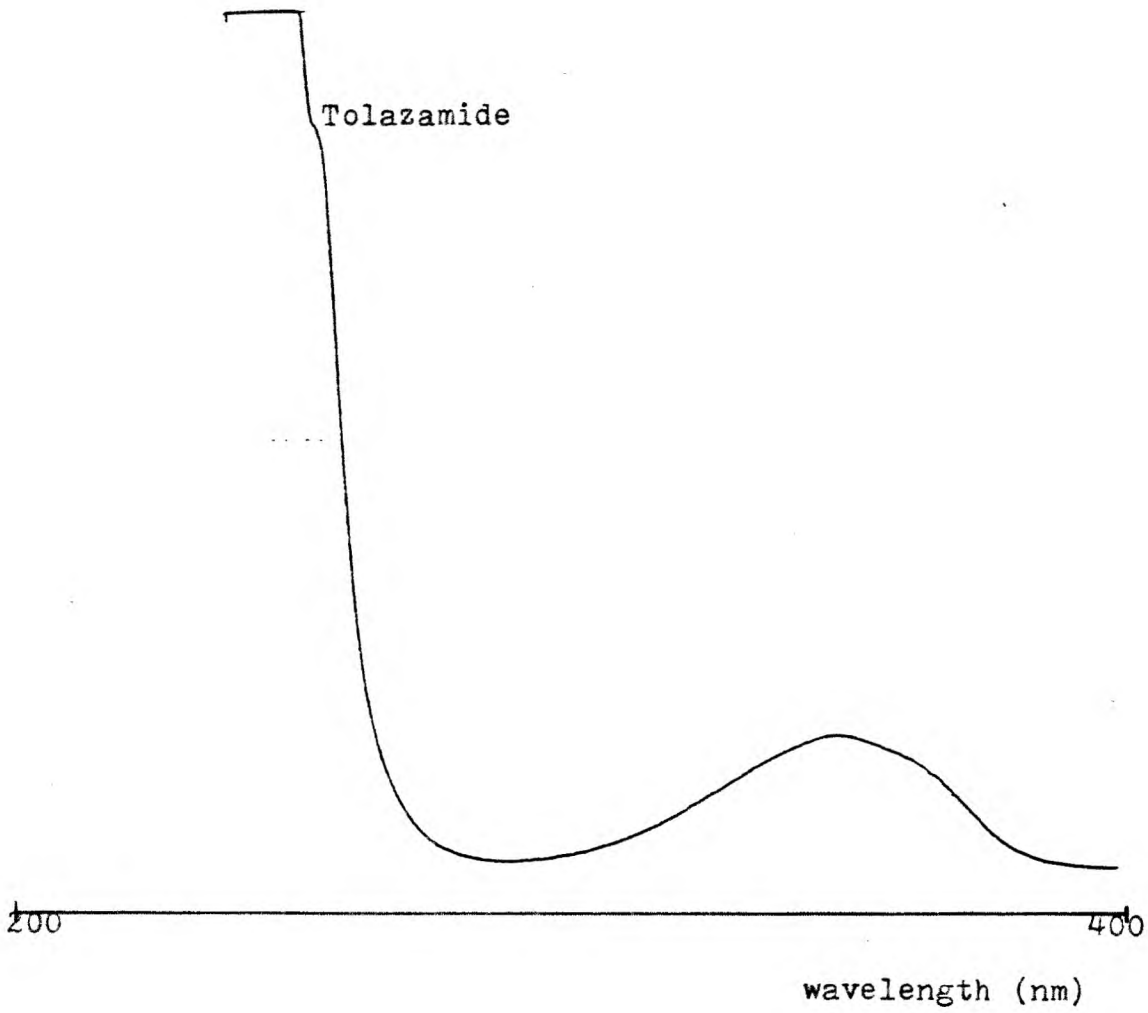


Figure 8. Ultraviolet Absorption Spectrum of a Mixture of Tolazamide and N-nitrosohexamethyleneimine

Figure 9. Ultraviolet Spectrum of the First Derivative of Absorbance Respect to Wavelength of a Mixture of Tolazamide and N-nitrosohexamethyleneimine

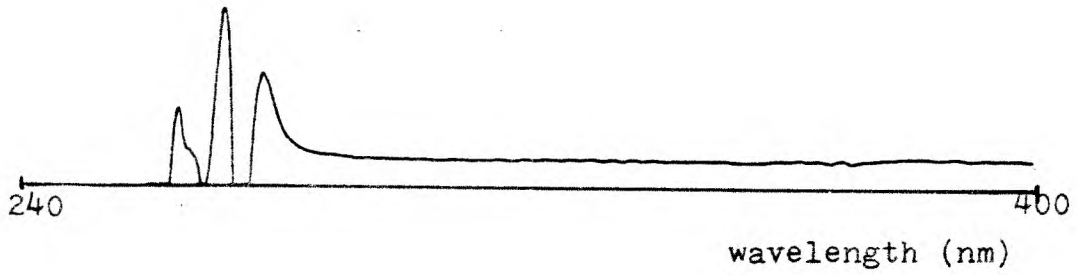


Figure 10. Ultraviolet Spectrum of the Second Derivative of Absorbance Respect to Wavelength of a Mixture of Tolazamide and N-nitrosohexamethyleneimine

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