High Performance Liquid Chromatographic Separation of Some Psychotherapeutic Benzodiazepines with Chemometrical Optimization

by

Aarif Hassan El-Mubarak

A Thesis Presented to the

FACULTY OF THE COLLEGE OF GRADUATE STUDIES

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN, SAUDI ARABIA

In Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

In

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This thesis, written by Aarif Hassan El-Mubarak under the direction of his thesis advisor and approved by his Thesis Committee, has been accepted by the Dean of the College of Graduate Studies, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

Thesis Committee

Prof. Salah M. Sultan (Advisor)

Dr. Abdullah A. Abdennabi (Member)

Dr. Nabil M. Fayad (Member)

Department Chairman

Dean, College of Graduate Studies

June 25th, 95

June 1995

To my Parents, wife and daughter

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THESIS ABSTRACT

FULL NAME OF STUDENT	**AARIF HASSAN EL-MUBARAK**
TITLE OF STUDY	HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SOME PSYCHOTHERAPEUTIC BENZODIAZEPINES WITH CHEMOMETRICAL OPTIMIZATION
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In this study, an accurate, selective, sensitive and nondestructive high performance liquid chromatographic (HPLC) method for the separation and quantitative determination of a mixture of some 1,4 benzodiazepines (namely Nitrazepam, Diazepam and Medazepam) was developed.

Using the modified simplex algorithm, together with a suitable chromatographic response function (CRF), an optimum condition of 89 % CH_3CN, 11 % 0.1 M acetate buffer, 3.44 ml/min flow rate, and 5 µl injected volume were obtained. Under these conditions the separation was achieved in less than two minutes. The effect of the experimental variables on the CRF was studied.

The method was found to be suitable for the determination of these compounds in proprietary drugs without suffering interferences from excepients. A recovery of 101.6 %, an accuracy of $\pm$ 0.11 ppm and a detection limit of 200 ppb were obtained. The developed method was superior to the existing standard method described for the determination of diazepam in drug tablets.

MASTER OF SCIENCE DEGREE

KING FAHD UNIVERSITY OF PETROLEUM AND MINERALS
Dhahran, Saudi Arabia

June, 1995

خلاصة الرسالة

أسم الطالب الكامل: عارف حسن المبارك

عنوان الدراسة: فصل بعض مركبات البنزودايازيبينات الدوائية بأستخدام طريقة الفصل الكروماتوغرافي و التحليل الأحصائي الكيمياتي الأمثل

التخصص: كيمياء تحليلية

تاريخ منح الدرجة: يونيو ١٩٩٥

تم تطوير طريقة دقيقة و حساسة بأستخدام الكروماتوغرافيا السائلة عالية الآداء لفصل و تقدير خليط من بعض مركبات بنزودايازيبين (بالتحديد مركبات نايترازيبام، دايازيبام و ميدازيبام). و قد تم التوصل الى أمثل ظروف الفصل و تحديد تركيزات المركبات الدوائية المختلفة بأستخدام طريقة سيمبلكس الأحصائية المعدلة و كانت عوامل الفصل المثلى كما يلي ٨٩٪ أسيتونايتريل و ١١٪ محلول أسيتات منظم و معدل تدفق قدره ٣,٤٤ مل/دقيقة وكان حجم العينة ٥ ميكروليتر٠ تحت العوامل المثلى السابقة تم فصل المركبات الثلاثة في أقل من دقيقتين و قد تمت دراسة المتغيرات التجريبية على دالة الأستجابة الكروماتوغرافية٠

و قد وجد أن الطريقة مناسبة للتقدير الكمي لهذه المركبات في المستحضرات الدوائية المختلفة دون تاثير يذكر من مكونات الدواء الأخرى٠ و كان مقدار الأسترجاع ١٠١,٦ ٪ و بمعدل دقة ± ٠,١١ جزء في المليون، و قد قدر الحد الأدنى لتركيز الدواء الذي يمكن تحديده بدقة كافية بمقدار ٢٠٠ جزء في البليون٠ تعتبر الطريقة التي تم تطويرها في هذه الدراسة أفضل بكثير من الطريقة القياسية المستخدمة للتقدير الكمي للدايازيبام في الحبوب الدوائية.

درجة الماجستير في العلوم

جامعة الملك فهد للبترول و المعادن
الظهران، المملكة العربية السعودية

يونيو ١٩٩٥

CHAPTER 1

INTRODUCTION

1.1 Benzodiazepines

1.1.1 Introduction

Benzodiazepines are psychotherapeutic agents which act on the central nervous system and have hypnotic, tranquilizing, anti-depressive, sedative, and anticonvulsant properties. It is an important class of drugs with different selectivity and varies in their usefulness, they are prescribed for the treatment of anxiety, sleep disturbance, and status epileptics, they are also used in the treatment of alcohol withdrawal and to relieve tension in the preoperative period and to induce amnesia in surgical procedures. In the last two decades, a large number of analytical and pharmacological studies of 1,4 benzodiazepines have been carried out. For the biopharmacological, clinical and toxicological studies of these drugs the availability of rapid, sensitive and selective analytical methods for their determination in pharmaceutical preparation and in the biological fluids is essential. The benzodiazepines are clinically effective at low doses ranging from 1 to 30 mg, resulting in blood concentrations in the 10-500 ng/ml range. They also undergo extensive metabolism and many of the metabolites are pharmacologically active; thus, it is essential that the assay methods be sensitive and specific *i.e.*, capable of separating and determining the parent drug as well as its major metabolites.

There are many techniques used to determine benzodiazepines in generic forms, pharmaceutical preparation and biological samples. These methods includes, UV spectrophotometric, polarography, thin-layer chromatography (TC), gas chromatography, among which high performance liquid chromatography (HPLC) methods are the most reliable, sensitive, efficient, and economic methods for the determination of these drugs in body fluids. HPLC offers several advantages over the other methods. The extraction procedures are relatively simple, and formation of derivatives are not necessary, and operation at ambient temperature allows the determination of benzodiazepines which is thermally unstable. The strong absorption in the 230-260 *n*m region gives sensitivity in the nanogram range and linearity over a wide concentration range. Furthermore, if the detection technique used is non-destructive, the eluted drug can be recovered for further examination.

1.1.2 Physico-chemical properties of Benzodiazepines

Benzodiazepines are chemically characterized by the presence of a phenyl ring fused to a saturated seven membered ring with nitrogen at the 1 and 4 positions. From their chemical structure, the most important types of 1,4 benzodiazepines are the following

- 2-keto derivatives, characterized by the presence of a C=O group at position 2 (Figure 1.1.2.1, structures A and C)

- 4-*N*-oxide derivatives, with *N*-oxide group at position 4 (Figure 1.1.2.1, structures B and C)

Other benzodiazepines not included in these major groups are presented in figure 1.1.2.1, structures D and L. The names and substituents of the most important

benzodiazepines drugs are listed in Table 1.1.2.1. Synthesis of benzodiazepines was investigated by many authors[1-3]. Benzodiazepines are basic in characters, and as neutral molecules they are soluble in organic solvents such as methanol, ethanol, diethylformamide and chloroform. They are slightly soluble in n-hexane or n-heptane and practically insoluble in water. The salt forms are water soluble[4]. Stock solutions of benzodiazepines in methanol and ethanol are stable for several weeks, and when they are stored in the dark at 4°C to -15°C, they are stable for several months. In aqueous or aqueous-alcoholic solutions, most benzodiazepines undergo hydrolysis, particularly under acidic or alkaline conditions, to produce benzophenone. The acid base characteristics of benzodiazepines are due to nitrogen atom at position 4, which can be protonated, except in 4-*N*-oxide-derivatives. The other nitrogen atom can be protonated. The hydroxyl group in the 3 hydroxy derivatives can be deprotonated at high pH, whilst the *N*-oxide group in 4-*N*-oxide-derivatives is protonated at low pH. Other relationships between structure and electrochemical properties, pharmacological activity have also been studied[5].

1.1.3 Reactions and metabolism

Analysis of benzodiazepines in pharmaceutical preparations is direct, provided that the separation conditions are well known, while the analysis of benzodiazepines in biological fluids requires a previous knowledge of the biotransformations that may occur. This is because the parent drugs as well as the derivatives will be existing together in the same complex matrix of the biological fluids. Metabolic reactions that different benzodiazepines can undergo are numerous, but some of the most important are the following:

1.1.3.1 Oxidation reactions

Oxidative reactions of many benzodiazepines take place in the liver by demethylation or dealkylation of the nitrogen in position 1, and hydroxylation of carbon in position 3

1.1.3.2 Conjugation

The 3-hydroxy substitution of some 1,4-benzodiazepines such as Oxazepam, Temazepam or Lorazepam allows direct conjugation to glucuronic acid, yielding pharmacologically inactive, water-soluble glucuronide conjugates that are excreted in the urine. Usually, an enzymatic hydrolysis is required in order to liberate benzodiazepines from their conjugates.

1.1.3.3 Nitroreduction

1,4-benzodiazepines having 7-nitro substituents, such as Nitrazepam, Flunitrazepam and Clonazepam, are biotransformed by reduction of the nitro group to form a biologically inactive 7-amino and 7-acetamido derivatives.

Another factor that should be considered is that, benzodiazepines are extensively bound to plasma proteins. This fact affects the blood level of the free drug, so always refer to free and bound fractions of the total drug amount in blood. A deprotienation process is required to liberate the bound fraction of the drug from the proteins.

1.2 Chromatographic Theory

1.2.1 Separation Process:

Chromatography involves the separation of the components of a mixture by means of differences in the equilibrium distribution K (sometimes called partition coefficient) of the components between two phases[6]: the mobile phase and the stationary phase. While in gas

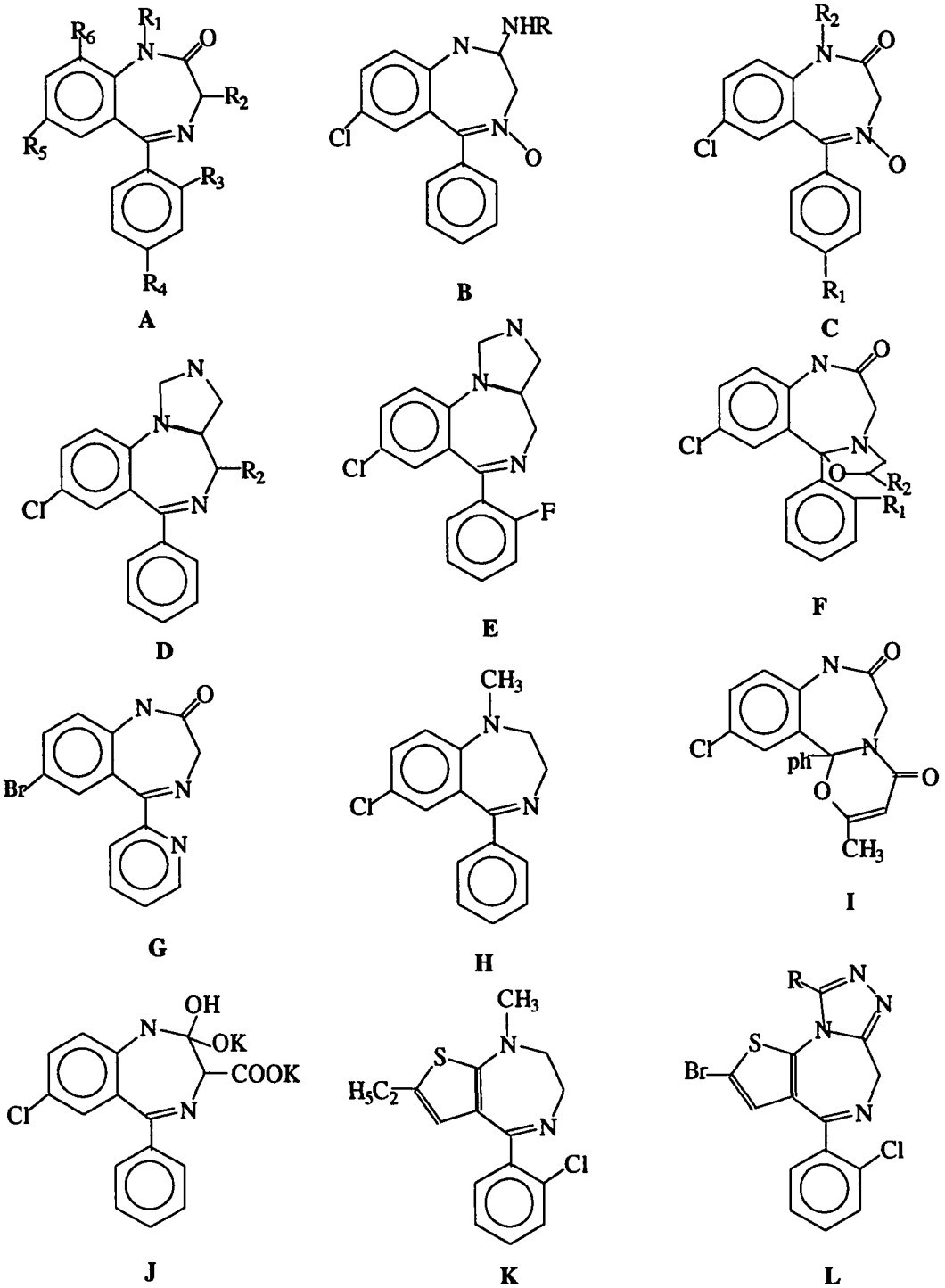


Figure 1.1.2.1. Chemical structure of some 1,4-benzodiazepines and related compounds. B (R=-CH3): Chlordiazepoxide. B (R = -H): Desmethylchlordiazepoxide. E (R= -CH_2OH): 1-Hydroxymethylmidazolam. G: Bromazepam. H: Medazepam. I (R = -CH3): Ketazolam. J: Clorazepate. K: Clotiazepam. L: (R = -CH3) Brotizolam.

TABLE 1.1.2.1. Names and substituents of some 1,4-benzodiazepines with relation to the structure shown in figure 1.1.2.1

	Structure A					
Name	**R_1**	**R_2**	**R_3**	**R_4**	**R_5**	**R_6**
Diazepam	$-CH_3$	-H	-H	-H	-Cl	-H
Temazepam	$-CH_3$	-OH	-H	-H	-Cl	-H
Oxazepam	-H	-OH	-H	-H	-Cl	-H
Nordazepam	-H	-H	-H	-H	-Cl	-H
Flunitrazepam	$-CH_3$	-H	-F	-H	$-NO_2$	-H
7-Aminoflunitrazepam	$-CH_3$	-H	-F	-H	$-NH_2$	-H
7-Acetamidoflunitrazepam	$-CH_3$	-H	-F	-H	$-NHCOCH_3$	-H
Norflunitrazepam	-H	-H	-F	-H	$-NO_2$	-H
3-Hydoxyflunitrazepam	$-CH_3$	-OH	-F	-H	$-NO_2$	-H
7-Amino-norflunitrazepam	-H	-H	-F	-H	$-NO_2$	-H
Lormetazepam	$-CH_3$	-OH	-Cl	-H	-Cl	-H
Lorazepam	-H	-OH	-Cl	-H	-Cl	-H
Delorazepam	-H	-H	-Cl	-H	-Cl	-H
Flurazepam	$-(CH_2)_2NHEt$	-H	-F	-H	-Cl	-H
N-1-Desalkylflurazepam	-H	-H	-F	-H	-Cl	-H
Monodesethylflurazepam	$-(CH_2)_2NHEt$	-H	-F	-H	-Cl	-H
Didesethylflurazepam	$-(CH_2)_2NH_2$	-H	-F	-H	-Cl	-H
N-1-Hydroxyethylflurazepam	$-CH_2CH_2OH$	-H	-F	-H	-Cl	-H
N-1-Desalkyl-3-Hydroxyflurazepam	-H	-OH	-F	-H	-Cl	-H
N-1-Flurazepam acetic acid	$-CH_2COOH$	-H	-F	-H	-Cl	-H
Clonazepam	-H	-H	-Cl	-H	$-NO_2$	-H
3-Hydroxyclonazepam	-H	-OH	-Cl	-H	$-NO_2$	-H
7-Aminoclonazepam	-H	-H	-Cl	-H	$-NH_2$	-H
7-Amino-3-hydroxyclonazepam	-H	-OH	-Cl	-H	$-NH_2$	-H
7-Acetamidoclonazepam	-H	-H	-Cl	-H	$-NHCOCH_3$	-H
7-Acetamido-3-hydroxyclonazepam	-H	-OH	-Cl	-H	$-NHCOCH_3$	-H
Nitrazepam	-H	-H	-H	-H	$-NO_2$	-H
7-Aminonitrazepam	-H	-H	-H	-H	$-NH_2$	-H
7-Acetamidonitrazepam	-H	-H	-H	-H	$-NHCOCH_3$	-H
Parazepam	$-CH_2-\Delta$	-H	-H	-H	-Cl	-H
Fludiazepam	$-CH_3$	-H	-F	-H	-Cl	-H
Nimetazepam	$-CH_3$	-H	-H	-H	$-NO_2$	-H
Camazepam	$-CH_3$	$OCON(CH_3)$	-H	-H	-Cl	-H
N-Deoxy-9-hydroxydemoxepam	-H	-H	-H	-H	-Cl	-OH
Pinazepam	$-CH_2-C\equiv CH$	-H	-H	-H	-Cl	-H
Bromazepam	-H	-H	-H	-H	-Br	-H
Medazepam	$-CH_3$	-H	-H	-H	-Cl	-H

** After L.A. Berrueta*

chromatography, the mobile phase is a gas, in liquid chromatography, the mobile phase is a liquid. In high performance liquid chromatography (HPLC), if C_s and C_m are the concentrations of a component in the stationary and mobile phases respectively, then:

$$K = \frac{C_s}{C_m} \tag{1-1}$$

Migration of component molecules may be assumed to occur only when the molecules are in the mobile phase. The rate of migration of a component then is inversely proportional to its distribution coefficient, so components with a high distribution in the stationary phase will move more slowly in the column and hence be separated from the components with a lower distribution in the stationary phase. Without this difference in distribution and, by inference, a differential rate of migration, no separation can be achieved.

Differential migration therefore depends upon the experimental variables that affect the distribution, i.e. the composition of the mobile and stationary phases, the flow rate, and the temperature. The column pressure effect on the distribution coefficient is usually negligible.

1.2.2 Retention in Liquid Chromatography:

With respect to the retention, the chromatographic zones move through the column at a rate less than the mobile phase velocity. The rate of the two velocities is known as the retardation factor

$$R = \frac{\text{rate of movement of the sample band}}{\text{rate of movement of the mobile phase}} \tag{1-2}$$

The elution time at peak maximum is known as retention time t_R, which can be related to the equilibrium distribution coefficient. It is a function of the mobile phase velocity and the volume of the mobile phase required to elute a component from the column. The retention volume (V_R) is given by:

$$V_R = F x\, t_R \tag{1-3}$$

where F is the volume flow rate of the mobile phase. The retention time of unretained peak, t_m, can be related to the volume of the column available to the mobile phase by:

$$V_m = F x t_m \tag{1-4}$$

In chromatographic theory, the occurrence of an elution peak with a Gaussian distribution (symmetrical peak) in the chromatogram is taken as showing a linear relationship between the concentration of the sample molecules in the stationary and the mobile phases; i.e. the distribution coefficient (K) is a constant and the isotherm is linear. Under these conditions the retention time is independent of the sample size. Skewed peaks are the result of non-linear distribution isotherm, and the retention time will vary with the sample concentration. If the sample is sufficiently small, it should be noted that the symmetrical Gaussian peaks can be obtained even for a component with a non-linear isotherm, and times will again be independent of the sample size. The fundamental retention equation for partition chromatography, neglecting non-linearity and band broadening, is:

$$V_R = V_m = K V_s \tag{1-5}$$

where V_R is the retention volume, V_m is the volume of the mobile phase, V_s is the volume of stationary phase, and K is the equilibrium distribution coefficient. A basic chromatographic parameter is the capacity factor k' where:

$$k' = \frac{n_s}{n_m} \tag{1-6}$$

and n_s and n_m are the number of moles of solute in the stationary and mobile phase respectively. This equation can be written in terms of retention time as:

$$k' = \frac{t_R - t_m}{t_m} \tag{1-7}$$

or in terms of retention volume as

$$k' = \frac{V_R - V_m}{V_m} \tag{1-8}$$

1.2.3 Band Broadening

When the sample molecules traverse into the column, the width of the band increases and the solute is diluted by the mobile phase. Three main contributions to band broadening are eddy diffusion, molecular diffusion, and mass transfer. In liquid chromatography, the eddy diffusion and molecular diffusion are not as important as mass transfer, which affects both the stationary and mobile phase

1.2.3.1 Stationary phase mass transfer:

The rate at which solute molecules transfer into and out of the stationary phase makes a significant contribution to band broadening. This rate depends mainly on diffusion of the liquid stationary phase and on adsorption - desorption kinetics for solid stationary phase.

1.2.3.2 Mobile phase mass transfer:

(a) Moving mobile phase mass transfer in which molecules in the same flow path will not all move in the same speed.

(b) Stagnant mobile phase mass transfer: When porous stationary phases are used, the intraparticle void volume is filled with mobile phase at rest. Solute molecules must diffuse through this stagnant mobile phase in order to reach the stationary phase, again resulting in broadening of the chromatographic band.

1.2.4 Plate height equation:

The column efficiency or number of theoretical plates, N, of a chromatographic system, is another important chromatographic parameter which may be defined from a single chromatographic band as follows,

$$N = 25\left(\frac{V_R}{W}\right)^2 \tag{1-9}$$

where V_R is the uncorrected retention volume and W is the peak width at the base line measured in units of time. This method is adopted throughout this thesis in calculating the peak width for integration and quantitation purposes, and that is because resolution is most critical near the peak base[7].

An equivalent expression which avoids the problem in measuring the basal peak width is:

$$N = 5.54\left(\frac{V_R}{W_{\frac{1}{2}}}\right)^2 \tag{1-10}$$

where $W_{1/2}$ is the peak width at half peak height. For a given set of operating conditions, the quantity, N, is approximately constant for different bands in the chromatogram and, is therefore, a measure of column efficiency. In comparing column efficiency, a more useful parameter is the height equivalent to a theoretical plate (HETP) or plate value H, where:

$$H = L/N \tag{1-11}$$

L is the length of the column and H measures the efficiency of the column per unit length. Small H values (large N values) therefore mean a more efficient column and this is one of the main goals in chromatography.

1.2.5 Resolution:

In a chromatographic system, the ability of a particular stationary phase or mobile phase to produce the separation is a function of the thermodynamics of the system. The solvent efficiency in terms of relative retention (α) for two components can be quantied as follows:

$$a = \frac{V_{R2}}{V_{R1}} = \frac{V_{R2} - V_m}{V_{R1} - V_m} = \frac{k'_2}{k'_1} = \frac{K_2}{K_1} \tag{1-12}$$

The relationship $\alpha = K_2/K_1$ emphasizes the thermodynamic nature of α. It can be shown that:

$$\Delta(\Delta G^{\theta}) = -RT \ln \alpha \tag{1-13}$$

where Δ (ΔG^{θ}) is the difference in free energies of distribution of the two components.

The combined effect of solvent efficiency and column efficiency is expressed in the resolution Rs of the column.

$$R_s = \frac{2(V_{R2} - V_{R1})}{W_1 + W_2} \qquad (1\text{-}14)$$

Purnell[8] have shown that for column chromatography the resolution (R_s) can be related to the capacity factor, (k'), the relative retention or selectivity, (α), and the number of theoretical plates (N) in terms of the second component (subscript 2) of the pair by the relationship:

$$R_s = \frac{1}{4}\left(\frac{a-1}{a}\right)\left(\frac{k'_2}{1+k'_2}\right)(N_2)^{\frac{1}{2}} \qquad (1\text{-}15)$$

To investigate further the effect that these terms have on the resolution, it is useful to consider them as independent functions.

1.2.5.1 Resolution and time of analysis:

Analysis time is not necessarily an important criterion. However, since resolution and analysis time are interrelated, plates per second (N/t) is a good criterion for comparing column performance. The time for analysis is approximately equal to the retention time of the last component. The retention time can be written:

$$t_R = N(1+k')(H/u) \qquad (1\text{-}16)$$

where υ is the mobile phase velocity. The use of higher flow rates is usually limited by practical considerations. The flow rate is related to the pressure drop through the column

(ΔP), column length L, mobile phase viscosity (η), and column permeability (K°), by the expression:

$$\upsilon = \Delta P K^\circ / \eta L \qquad (1\text{-}17)$$

1.3 Optimization in HPLC:

1.3.1 Introduction:

Optimization methods in analytical chemistry are of great benefit if the experimental variables are identified. Yet optimum values are not always uniquely establishiable and not transferable between laboratories nor between individual experimental set-ups and times. While this is in principle the case for most analytical procedures, it is particularly annoying in high performance liquid chromatography because of the range of selectivities that can be achieved through minor changes of the mobile phase and frequently poor reproducibility of column parameters, like resolution and surface chemistry of the stationary phase.

Development of high performance liquid chromatographic (HPLC) methods can be a very time-intensive task for the chromatographer. In the development process, the column, mobile phase, flow rate, and other factors can be varied to improve separation quality. In recent years, several systematic approaches to optimizing some of these variables have been proposed and automated[9]. These approaches optimize mobile phase composition, and in some cases, flow rate once the choice of column has been made. All of these methods evaluate chromatograms using a variety of mobile phases until an optimal separation for that particular column has been achieved.

In order to automate the method development task, a mean of numerically quantitating the quality of a separation is necessary. A chromatographic response function (CRF) is the most widely method of translating chromatographic information to a numerical representation, and several such functions have been used (Table 1.3.1.1.)[10]. The success of an optimization depends largely on how well the CRF quantities the factor by which chromtographers intuitively judge separation. The three primary factors are peak separation (in terms of resolution), analysis time, and the number of peaks. Each of these factors varies in importance depending on the application of the method.

There is an increasing number of schemes being developed to aid in the optimization of high performance liquid chromatography separations[11]. Overlapping resolution mapping[12,13], window diagrams[14,15], iterative mixture design [16-18], and the sequential simplex procedure[19,20] are the most commonly used. Other procedures including iterative methods are also used. In this work, the modified simplex algorithm is applied throughout the optimization process.

TABLE 1.3.1.1. Chromatographic optimization functions used with simplex optimization.

Function	***Variables***
$P_{inf} = \sum_i {}^2 \log S_i$	Binary mobile phase
$F_{obj} = \sum [10(1.5 - R_i)]^2$	Ternary mobile phase
$F_{obj} = \sum 100e^{1.5-Ri} + (t_m - t_n)^3$	Ternary mobile phase
$CRF = \sum ln(P^i/P_d) + a(t_m - t_n)$	Gradient parameter and flow rate binary mobile phase
$CRF = \sum ln(f_i/g_i) - 100(M - n)$	Concentration of organic modifier, pH
$CRF = \sum R_i + n^a - b\lvert t_m - t_n \rvert - c(t_0 - t_1)$	Composition of ternary mobile phase, temperature, flow rate, pH
$CRF = \sum R_i + n - c(t_m - t_n)$ for $(t_m - t_n) > 1$	Composition of ternary mobile phase
$Y = p/M$	Gradient parameter (S/b)

P_{inf} = informing power, S_i = peak overlap, R_i (P_i) = actual resolution (peak separation) and R_d (P_d) = desired resolution (peak separation), t_n = retention time of last peak and t_m = desired retention time, t_0 = void time and t_1 = retention time of the first eluted peak and f and g = peak separation factors; N = noise, M = number of peaks expected, n = number of peaks detected and P = number of peaks separated with a given resolution; CRF = chromatographic response function and Y = the extent of separation; the parameters a, b and c are selectable weightings.

1.3.2 Theory:

1.3.2.1 Simplex Algorithm

There are many variants of the simplex procedure, yet they all depend on the principle procedure of Spendley *et al.*[21]. A simplex is defined as a geometric figure in which the number of initial experiments conducted is one more than the number of parameters (*i.e.* mobile phase combination, flow rate temperature, etc) to be simultaneously optimized. The initial experiments establish the vertices of a geometric figure (simplex), which will move through the factor space in search of the optimum. When the preliminary simplex is established, the vertices with the lowest value is rejected, and a new point is found by reflecting the simplex in the direction away from the rejected vertex. It is a hill climbing method whose direction of advance is dependent mainly on the ranking of response, and in this way the simplex proceeds toward the optimum set of conditions. To accomplish this goal, certain rules were applied to direct the decisions. These rules were first suggested by Deming and Morgan[22], and later were modified by Nelder and Mead[23]. These rules are:

Rule 1: A move is made after each observation of response.

Rule 2: A move is made into that adjacent simplex which is obtained by discarding the point of the current simplex corresponding to the least desirable response and replacing it with its mirror image across the hyper face of the remaining points.

Rule 3: If the reflected point has the least desirable response in the new simplex, do not reapply rule 2, but instead reject the second lowest response in the new simplex and continue.

Rule 4: If the vertex has been retained in k + 1 simplex, before applying rule 2 reobserve the response at the persistent vertex.

Rule 5: If a new vertex lies outside the boundaries of the independent variables, do not make an experimental observation, but instead assign to it a very undesirable response. However, more details on the simplex algorithm and its mathematical derivations are available elsewhere[24-28]. Also more detailed descriptions of the process have been published recently[29,30].

Some advantages of the simplex method in the optimization of HPLC separation include the following:

(1) It is able to optimize many interdependent variables with no prior knowledge about the mode of separation or the complexity of the sample.

(2) It does not require any pre-conceived model of the retention behavior of the solutes.

(3) The method permits the introduction of new variables during the optimization process for the price of just one additional experiment per variable, and one can also assess the process of optimization during, rather than at the end of the experimental sequence.

(4) Computational requirements relative to other statistical methods are minimal.

(5) Any number of parameters may be considered.

However, some disadvantages of the simplex algorithm are:

(1) The ranking of responses requires that the quality of the chromatogram from each experiment be assessed.

(2) A large number of experiments may be required to find an optimum.

(3) A local rather than a global optimum may be found.

With respect to the latter deficiency, the chances of finding a global optimum are enhanced using a modified simplex, which allows other operation other than reflection such as expansions and contraction. The chances of mistaking a local optimum for the global optimum are also reduced by restarting the simplex in a different region of the parameter space.

1.4 Objectives

The objectives of the work are:

1. To determine the optimum separation conditions in a predetermined time of a mixture of some 1,4 benzodiazepines (namely, Diazepam, Medazepam, and Nitrazepam) optimization by HPLC using simplex procedure.

2. To develop a fast, accurate, and sensitive nonrestrictive method for the assay of benzodiazepines and their derivatives for pharmaceutical preparation which can be applied as well to biological samples if time allowed.

3. To confirm the separation and the method developed by verifying the figures of merit in order to see the validity of the method.

CHAPTER 2

LITERATURE REVIEW

2.1 Benzodiazepines

This literature review will highlight in brief some of the historical developments that have taken place in the field of benzodiazepines, the high performance liquid chromatography (HPLC) technique and the optimization of HPLC. The first synthesis of benzodiazepine, which was desigened on the basis of structure activity relationships work, led to flunitrazepam[31] (see the structure in chapter 1). By 1960, it has been established that the most potent substituent in the 7-position was the electron withdrawing nitro group; that the 2-fluorophenyl group was the most activating substituent in position 5, and that N-methylation of the amide nitrogen enhanced activity. Also, it is known that compounds containing the carbonyl oxygen of the amide group and compound that retained the Schiff's base double bond were more active than their counterparts[32]. Few years after, various modifications of the seven-membered ring were made, which showed that anxiolytic activity could be retained by rearranging the 1,4 diazepine structure. Thus it was discovered that the C, N double bond could be replaced by an amide group, which gave the 1,5-benzodiazepine-2,4-diones such as clobazepam, which was first synthesized by von Weber *et. al.*[33]. Another modification carried out by Golik[34,35] was the synthesis of the active 2,4-benzodiazepine-1-ones, in which the amide functionality at the 1- and 2-positions has been reserved. It was therefore apparent that the traditional "A" ring of the benzodiazepines could be any aromatic or heteroaromatic system and, although there was some freedom in terms of the order of the arrangement of the atoms in the diazepine part of the ring system, the 7-membered ring was essential for activity.

The early introduction, as of 1960, of chlordazepoxide (Librium™) open the door for a large number of 1,4 benzodiazepines to be investigated. Since that time, some reviews on this subject have been published [36-39]. Two books that contain some analytical data pertaining to 1,4-benzodiazepines have been edited [40,41], as well as a number of articles which reflect the importance of this family of drugs. Benzodiazepines in pharmaceutical preparations as well as in biological fluids are determined by various techniques, of which high performance liquid chromatography (HPLC)[42-54], gas-liquid chromatography (GLC)[55,66], and polarography[57,88] are the most dominant. Although the GC and GLC methods are extremely sensitive, they require lengthy clean-up procedures and in some cases, formation of the derivatives. Moreover, if high temperatures are used, they may cause the decomposition of certain benzodiazepines such as chlodiazepoxide, oxazepam, lorazepam, and their metabolites. Polarography, despite the advantage of convenience and speed, lacks the sensitivity and specificity of GLC, and requires large amounts of sample. Other methods including electrochemistry and spectrophotometry have been recently reviewed[59].

2.2 High Performance Liquid Chromatography (HPLC)

With respect to HPLC theory and optimization, a number of general reviews, tutorials, and prospectives were published. Bellot and Condoret reviewed the different ways to express the adsorption isotherms, competitive or not, in reversed phase chromatography. Once the equilibria are described, powerful liquid chromatography models, based on continuity equation, can be employed to describe the phenomena encountered in the separation process of bimolecules[60]. Lundell reviewed the option in the implementation of gradient theory for the optimization of peptide separation in Reverse Phase Liquid Chromatography (RPLC), discussing approaches for calculating retention times and band widths from experimental data and comparing different kinds of extrapolation with

interpolation. The study was aimed at finding the best compromise between number of experiments, accuracy of prediction, and simplicity of calculations[61]. Scott discussed the role of molecular interactions and molecular size in controlling separation in the context of the preparation of the stationary phase[62]. Band dispersion is discussed in terms of multipath effect, longitudinal diffusion, and resistance to mass transfer, and the basic component of the modern LC chromatograph are examined. Volpe and Siouffi reviewed (38 references) some of the problems in gas, liquid, and supercritical fluid chromatography[63]. The authors challenged that HPLC suffers from lack of a reliable model to predict the capacity factors for a wide range of solute, that retention mechanisms on some phases are not fully understood, and the diffusion coefficient data are scarce.

A theory by Roach describing the overlap of circles in two dimensional plane was elegantly generalized to an *n*-dimensional space, where *n* is the number of orthogonal axes. The statistical theory of spot overlap is proposed for *n*-dimensional separation. Extensive computer simulations that the theory describe overlap very well in three dimensional space and modestly in four dimensional spaces when the number of component is large. The theory shows that the maximum number of spots per unit capacity and the maximum number of any kind of multiplet per unit capacity both decrease geometrically with increasing *n* [64]. Liapis and McCoy constructed a mathematical model of perfusion chromatography for column system[65]. The model was developed to describe the dynamic behavior of single and multicomponent adsorption in columns having perfusive adsorbent particles, but was also found to be applicable to columns with diffusive particles, *i.e.* one in which inter particle fluid velocity is zero. For an adsorption system having relatively fast or infinitely fast interaction kinetics (*i.e.* analyte-stationary-phase dynamics), the use of perfusive particles can potentially provide improved column performance. Later, McCoy et. al. use a mathematical model of binary perfusion chromatography (competitive adsorption involving two components) to simulate, study,

and compare the separation of two components with perfusive and purely diffusive particles[66]. Jandera and Guiochon investigated the dependence in preparative HPLC of band deformation and splitting on the volume and concentration of the sample, the composition of the mobile phase, and the column temperature. They showed that if a solvent with a higher elution strength than the mobile phase is used to dissolve poorly soluble samples, significant deformation of the band profile occurs, specially when the column is overloaded; eventually, band splitting may take place. This behavior was observed in monoaquous RPLC of cholesterol and other low polarity analyte [67].

Belenkii and co-workers described some peculiarities of zone migration and band broadening in the gradient RPLC of proteins with respect to membrane chromatography[68]. Blackwell and Carr developed an elutropic series for the separation of Lewis bases on zirconium oxide[69]. Dreyer et. al. demonstrated that the steric exclusion effects can have a far greater influence on retention data than is commonly assumed [70]. Gloeckner discussed the control of adsorption and solubility in HPLC separation[71]. Hsu and Chen reported that a conventional definition of column efficiency is inadequate for large scale liquid chromatography with small equilibrium constants[72]. Liu and co-workers realized all the predicted advantages of high temperature in liquid chromatography[73]. Slais te. al. suggested the separation of analytes in capillary, providing a mathematical description of retention under certain conditions[74,75]. Wankat developed a method for scaling chromatographic systems[76].

2.3 HPLC Optimization

Destefano and co-workers described RPLC method development based on column selectivity[77]. Gustavo-Gonzalez and Asuero applied the extend Hildebrand solubility

parameter treatment to optimize RPLC determination of pharmaceuticals[78]. Hamoir et. al. constructed models for the prediction of initial chromatographic conditions in pharmaceutical analysis by RPLC [79]. Heinish and co-workers described several prioritized criteria when searching for the best eluent strength using interpretive methods[80]. Iwaki et. al. utilized an information theory-based chromatographic response function to select a stationary phase that provides the best precision of peak shape and overlap[81]. Palasota and co-workers described the application of the sequential simplex algorithm for improved separation of amino acids in a constrained simplex mixture space[82]. Vanbel et. al. discussed chemometric optimization in drug analysis, providing a critical evaluation of the quality criteria used to determine drug purity[83]. Wieling and co-workers performed robustness testing of an optimized RPLC system for the separation of sulfonamides using the rules of error propagation[84]. Wilce et. al. applied experimentally derived retention coefficients to the prediction of peptide retention times, focusing on myohemerythrin[85]. Among all chemometrical procedures used to optimized HPLC for normal or reversed phase, isocratic or gradient elution, simplex algorithm showed a great popularity. This is mainly due to the advantages mentioned previously.

CHAPTER 3

EXPERIMENTAL

3.1 Instrument

The apparatus which has been used for all chromatographic separations through out this work is a WATERS / USA system which consist of the following units:

- Waters 600E Multisolvent Delivery System.
- Waters Intelligent Sample Processor (WISP) Model 712 .
- Waters 990 Series Photodiode Array Detector.
- NEC Power mate 2 Data Statio. equipped with Waters 5200 Printer Plotter.

3.1.1 Waters 600E Multisolvent Delivery System

The Waters 600E Multisolvent Delivery System is a modular High Performance Liquid Chromatography solvent delivery system, which is capable of automatically selecting flow and gradient composition to blend up to four solvents. It is divided into two modules: (1) the fluid handling unit contains devices such as the pump, pressure transducer, solvent inlet manifold, sparge valve, and solvent proportioning valve which are required to deliver solvents from four solvent reservoir bottles to the injector. (2) the controller unit contains the electronics, firmware, and the power supply. These two modules are connected by a control cable. The pump is capable of mixing as many as four solvents (even in extreme proportions) with high reproducibility. The switching

valve construction produces precise gradient segments regardless of solvent compressibility and system back pressures.

3.1.2 WISP Model 712 Waters Intelligent Sample Processor

The Waters Intelligent Sample Processor (WISP™) is a versatile, automatic sample injection module for use in high performance liquid chromatography (HPLC) systems. Measured aliquots (1 µl to 2000 µl) can be programmed for up to 48 sample vials for injection onto a column while maintaining a continuous flowing mobile phase. The WISP can be operated to perform single injections or programmed for unattended operation combining various samples and standards. One to nine injections can be made from each vial with similar or varied injection parameters (injection volume sample run time and equilibration delay between runs). The sample handling method in the WISP includes an external needlewash system to prevent sample cross contamination without the necessity of placing needlewash vials in the sample carriage.

3.1.3 Waters 990 Series Photodiode Array Detector

The photodiode array detector is a UV/VIS spectrophotometer, and shares many features with a conventional UV/VIS detectors. In the conventional UV/VIS detectors, a single channel or scanning UV/VIS spectrophotometer separates light with a diffraction grating before the light reaches the sample. In a photodiode array detector, polychromatic light from the light source is transmitted through a lens and shutter directly to the sample in the flow cell[86]. The relationship between the quantity of light of a particular wavelength arriving at the photodiode and the concentration of the sample is described by the Lambert-Beer law, which applies only for well equilibrated dilute solutions. It assumes that the sample's refractive index remains

constant, the light is monochromatic, and that no stray light reaches the detector element. As concentration increases, the chemical and instrumental requirements of Beer's law may be violated, resulting in a deviation from linearity.

3.1.4 NEC Power mate 2 Data Station. equipped with Waters 5200 Printer Plotter

The NEC power mate 2 is a computer which is furnished with a 991 Waters photodiode array program necessary to control the operation of the HPLC.

3.2 chemicals and Reagents

3.2.1 Stationary phase

Different types of columns have been used with different mobile phase combination for the purpose of selecting one which gives better separation. These columns were obtained directly from manufacturing companies. All of them are either C18 or C8 bonded phase, but they differ in their length, diameters, and end - capping. Some of these columns include Supelcosil™ LC-8, 15cm x 4.6 mm id from Supelco, Lichrosorb™ RP-18 (C18), 10µ, 20 cm x 4.6 mm id from Hewlett-Packard, and µBondapak™ C18, 10µ, 30 cm x 3.9 mm id from water.

3.2.2 Mobile phase

All solvents used as mobile phase were of HPLC grade. Organic mobile phase solvents were filtered through 0.22 µm Millipore glass fiber filters. Aqueous mobile phases include organic-free water, which is triply distilled, filtered through 0.45 µm Millipore glass fiber filters before use . Buffers were prepared using the same grade of water.

3.2.3 Chemicals

Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium acetate, and all other chemicals were of HPLC grade used without any further purification.

3.2.4 Drugs

Pure drug samples were obtained as a gift from a drug house (Roche, Switzerland). Drug tablets which are commercially available were obtained from local drug store.

3.3 Procedures

3.3.1 Preparation of buffers

All glassware throughout this study were cleaned thoroughly with detergents, triple distilled and organic free water, acetone, methylene chloride, and heated overnight in the oven at 180 °C.

Ammonium acetate buffer was prepared by dissolving 15.416 g of CH_3COONH_4 in triple distilled and organic-free water. The volume was made up to 2000 ml to give a 0.1 M acetate buffer. The pH was adjusted to the desired value using acetic acid at 25° C. Phosphate buffers were prepared from two solutions, Stock solution 1 of 0.1 M K_2HPO_4 was prepared by dissolving 17.418 g of K_2HPO_4 in one litter of triple distilled and organic-free water. Stock solution 2 of 0.1 M KH_2PO_4 was prepared by dissolving 13.609 g of KH_2PO_4 in one liter of triple distilled and organic-free water. The phosphate buffer with a pH 8.0 at 25°C was prepared by adding 47.35 ml of 0.1 M K_2HPO_4 to a 2.65 ml of 0.1 M KH_2PO_4 and diluted to 100 ml with H_2O. Higher buffer volumes were prepared by suitable dilution in the same fashion[87].

3.3.2 Preparation of stock solutions of 1,4 Benzodiazepines

In three clean amber 100 ml volumetric flasks, amounts of 0.1002g Nitrazepam, 0.1001g Diazepam and 0.1002g were weighed, the volume was made up by CH_3CN (filtered through 0.22 µm glass fiber Millipore filter) to give a concentration of 1.0 mg/ml of each compound. Further mixtures and working standard with different concentrations were prepared by appropriate dilution.

3.3.3 Mobile phase preparation

Acetonitrile, methanol, tetrahydrofurane, buffers, and water were filtered through an appropriate filter and degassed by sparging all mobile phase solvents with a helium gas at a rate of 100 ml/min for one hour, then kept at a constant sparging rate of 20 ml/min through out the analysis. This practice was done daily as a routine for the system conditioning before the analysis. Degassing solvents used in the mobile phase is one of the most effective measures applied to eliminate these problems. The benefits are: stability in the baseline and enhanced sensitivity in some type of chromatographic detectors, reproducible retention times for eluting peaks, reproducible inject volumes for quantification, and stable pump operation. Sparging, or bubbling a gas through solvent is the most used method among others which include heating, vacuuming and sonication. Sparging reduces the partial pressure of the unwanted gas on the surface of the solvent. This removes unwanted gas from the solution.

3.3.4 Column conditioning

Stationary phase or column was conditioned at the beginning and before start by passing first, a mobile phase of methanol:water 50:50 at a flow rate of 1-2 ml/min for 30-60 min, then acetonitrile:water 50:50 at a flow rate of 1-2 ml/min for 30 min, then the combination of the mobile phase used for the method development and analysis. This practice will remove uneluted compounds which have been left behind from previous runs, if any. Also, it will refresh the column by regenerating the adsorption active site, leaving the column ready for the next run, and it will give a chance for a steady flow of the desired combination of the mobile phase after measuring the flow at the end-tubing before going to the waste.

3.3.5 Detector conditions

Photodiode array detector (PDA) is stable and sensitive enough that, a warm up for 5-10 min is quite sufficient for the UV light to condition the optics, the flow cell, and the diode array as well as the electronics. Sampling time, which is the amount of time taken for the entire photodiode array to be discharged by light, was let to be automatically set by the PDA, giving 80-90% reading of absorbance at full scale. For routine analysis, it is not necessary to manually adjust the sampling time. If working in a range other than the optimum UV range, or must compensate for an aging lamp, sampling time should be adjusted so that the peak wavelength of interest is within 80 to 90 percent of full scale. Adjusting the sampling time may cause the PDA to automatically adjust the interval which determines how often data is collected. Accumulation, which is the averaging of several scans of the sample into one data point, was set by the PDA based on the sampling time, resolution and interval. Acquisition mode reflects the number of data points stored per second during collection. The PDA set the acquisition mode based on sampling time, resolution, and

interval. The sense which controls a rolling average of accumulated data on the time axis was adjusted by the PDA. This parameter filters the noise in low concentration ranges. The wavelength range is adjusted to read 240-285 nm with a resolution of 1 nm. This range represents the practical range for the commonly known 1,4 benzodiazepines, which absorb UV strongly in this region. The wavelength of 254 nm was chosen as a compromised wavelength for the selected drugs.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

4.1.1 Developing a chromatogram

In this chapter the analytical conditions and methods for the separation and the quantitation of Nitrazepam, Diazepam and Medazepam, as applied in this study, are discussed. Although most liquid chromatographic problems can be solved by more than one mode of liquid chromatography, the final approach may depend on the availability of equipment, columns, or personal preferences of the chromatographer. The isocratic reverse phase high performance liquid chromatography RP-HPLC was adopted throughout this work. Since one of the major objectives of this work is to develop a method for the separation of the above mentioned benzodiazepines, this method should be analytically applicable for clinical, pharmaceutical, as well as research and development analysis. All methods reported in the literature up-to-date lack the required speed. The most successful available methods are in the range of 5-8 min for two of the above three compounds, namely Nitrazepam and Diazepam. To be applicable for a routine clinical laboratory, the method should be adequately fast if not rapid, thus in addition to separation, the time factor is important in this aspect. Isocratic mode best suits this problem, rather than gradient elution, since it is less time consuming, in terms of conditioning of the gradient shape and column. Optimization of chromatographic parameters, which is the second objective of this work, is the only way to achieve the

desired separation and to meet the time domain limitation which is already predetermined to be two minutes, and which was thought to be enough for the clinical routine analysis, giving a reasonable throughput frequency of 20-30 samples per hour. Throuhput will depend on the auto injection technique, and the injection delay time between the samples.

After the selection of the chromatographic mode, the selection of the column was limited by the suitability and availability of the columns. A C18-silica bonded phase is the right choice. Three C18 columns are available for the analysis, namel, Supelcosil™ LC-8, 15cm x 4.6 mm id, from Supelco, Lichrosorb™ RP-18 (C18), 10μ, 20 cm x 4.6 mm id from Hewlett-Packard, and μBondapak™ C18, 10μ, 30 cm x 3.9 mm id from Waters. A 10 ppm standard mixture of Nitrazepam, diazepam, and Medazepam each, was used throughout the separation, optimization and calibration. Preliminary investigation was made with each column and after conditioning, a different combination of a single, binary, or tertiary mobile phase containing methanol, acteonitrile, tetrahydrofuran (THF) and water was tried in different proportions with 10-20 % step ranges to see which one gave the best separation. The combination of acteonitrile and water with Lichrosorb™ RP-18 (C18), 10μ, 20 cm x 4.6 mm id from Hewlett-Packard gives adequate separation compare to the other mobile and stationary phases.

4.1.2 Chromatographic response function (CRF)

In order to assess the quality of a chromatographic separation, a mean of quality criterion is needed. This criterion should be a single numerical value and of a magnitude that describes the separation very well. Also the criterion should lead towards a better separation. Many criteria have been used depending on the objectives of the separation and optimization. The Chromatographic Response Function (CRF) is the most widely

used quality criterion. The CRF which has been used throughout this work is that expressed by Wright and Fell[88] as follows:

$$CRF = \sum_{i=1}^{n-1} R_i + n - |T_A - T_L| \qquad (4\text{-}1)$$

This expression incorporates the summed resolution between adjacent peak pairs (R_i), the number of peaks detected (n), and a target retention time for the last peak, (T_A). However, a computer program has been written in BASIC for the calculation of the CRF, including all necessary equations. The program ensures that any retention time for the last peak, T_L that occurs within ±1 minute of the target time, T_A, is equally acceptable, since the last term in the CRF is only included if it exceeds 1 minute. The target retention time used in the present work is 2 minutes. The resolution was taken as:

$$R_s = \frac{1}{4}\left(\frac{a-1}{a}\right)\left(\frac{k'_2}{1+k'_2}\right)(N_2)^{\frac{1}{2}} \qquad (4\text{-}2)$$

where:

$$k' = \frac{V_R - V_m}{V_m} \qquad (4\text{-}3)$$

and

$$a = \frac{V_{R2}}{V_{R1}} = \frac{V_{R2} - V_m}{V_{R1} - V_m} = \frac{k'_2}{k'_1} = \frac{K_2}{K_1} \qquad (4\text{-}4)$$

and

$$N = 25\left(\frac{V_R}{W}\right)^2 \qquad (4\text{-}5)$$

where k' is the capacity factor, α is the selectivity between two peaks, N is the number of theoretical plates for each peak, V_R is the retention volume, and W is the peak width at base. The retention volume is a factor of the flow rate and retention time

$$V_R = F \times t_R \qquad (4\text{-}6)$$

where F is the flow rate and t_R is the retention time of a specific compound (peak).

4.2 Optimization of [H+]:

After the selection of the CRF, and developing the computer program for the CRF calculation, a search for the best pH buffer started using a simplex algorithm to search for the optimum condition, first for pH, and later for the method development. Due to the fact that in aqueous, aqueous-alcoholic, or aqueous-acetonitrile solutions, most 1,4 benzodiazepines undergo hydrolysis, particularly under acidic or alkaline conditions, the use of buffer in the mobile phase is exceptionally critical and important at a fixed pH value to prevent the derivatization or transformation of the parent drug. Depending upon the different conditions and the 1,4 benzodiazepines type, hydrolysis can affect the 3,4-azomethine group, the 1,2-amidic bond, or both, producing the corresponding benzophenone[89]. In most of the work reviewed, the mobile phases consist of a mixture of acetonitrile or methanol (or both), and water or aqueous acetate or phosphate buffer at weakly acidic pH depending on the stationary phase and type of benzodiazepines. First a

phosphate buffer was used in a pH range of 6.0-8.0, covering a weakly alkaline to a weakly acidic range. The results does not show any improvement in the separation process. Then an acetate buffer was used with a pH range of 3.6-6.0, which remarkably showed a significant improvement in the separation process at pH 6.0. Table 4.2.1. lists the boundary conditions and simplex optimization when only acetonitrile and water were used (no buffer). The results shows a local optimum at a CRF value of 19.96 with a diffused peak for the last compound. Figure 4.2.1 shows the simplex movement during the optimization of HPLC parameters using H_2O in the mobile phase.

The separation of the three compounds using 0.1 M acetate buffer at a pH of 3.6 and with a lower boundary condition of 50% showed even a poorer separation than using H_2O alone. This is mainly attributed to the increase of the aqueous portion in the mobile phase. Table 4.2.2 lists the boundary conditions and simplex optimization when acetate buffer at a pH of 3.6 was used. Figures 4.2.2 and 4.2.3 (a, b, and c)shows the simplex movement during the optimization of HPLC parameters and typical chromatograms of selected vertexes during the optimization. Higher pH (4.7) of the acetate buffer was used in order to improve the mobile phase strength, and hence the separation, by narrowing the aqueous mobile phase boundaries. This result is an improvement of the simplex progress with a higher value of CRF of 23.61 at vertex 18. Table 4.2.3. lists the results of this setup while figures 4.2.4. and 4.2.5 (a, b and c) show the simplex movement during the optimization of HPLC parameters and typical chromatograms of a selected vertexes during the optimization. The improvement in the simplex progress gave a greater chance for a weakly acidic pH to be selected. The results of the simplex optimization using 0.1 M acetate buffer with a pH of 6.0 gave the best separation. The optimum was recorded at vertex 19 with a CRF value of 26.403. The simplex movement was leveling toward the optimum in an increasing fashion with a minima at vertex 10.

TABLE: 4.2.1 Boundary conditions and simplex optimization for HPLC variable using H_2O (No buffer) in the mobile phase for the mixture of Benzodiazepines (10 ppm each).

Boundary conditions:

Variable:	Upper	Lower	Step	Start
% CH_3CN	100	50	10	75
% H_2O	50	0	10	25
Flow rate	4.0	1.0	0.6	2.0

Experiment No.	% CH_3CN	% H_2O	Flow rate mil/min	CRF
1	85.00	15.00	2.00	13.01
2	78.96	21.04	2.05	13.10
3	83.38	16.62	2.19	14.35
4 R	77.35	22.66	2.25	13.04
5 R	81.77	18.24	2.39	14.65
6 E	83.17	16.83	2.55	15.64
7 R	89.20	10.80	2.50	15.33
8 R	88.99	11.01	2.86	15.93
9 R	82.95	17.05	2.91	17.49
10 C	84.57	15.43	2.72	15.49
11 R	77.13	22.87	2.61	18.58
12 R	76.91	23.09	2.97	19.96

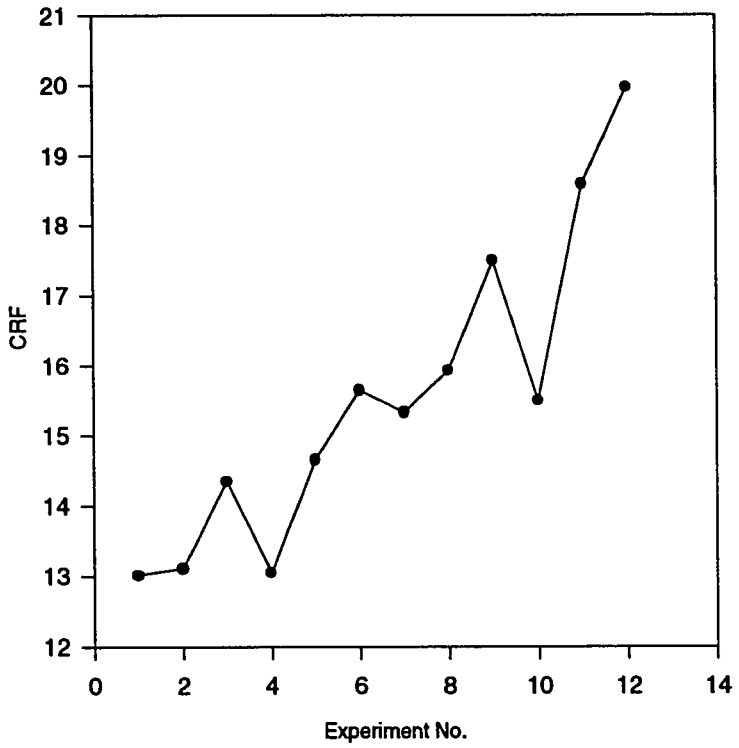


Figure 4.2.1 Simplex progress using H_2O in the mobile phase

Table 4.2.4. lists the boundary conditions and simplex optimization when acetate buffer at a pH of 6.0 was used. Figures 4.2.6. and 4.2.7.a, b and c shows the simplex movement during the optimization of HPLC parameters and typical chromatograms of a selected vertexes during the optimization. From the above results, the 0.1 M acetate buffer with a pH of 6.0 was chosen to be the second mobile phase eluent in the binary mobile phase system.

TABLE: 4.2.2 Boundary conditions and simplex optimization for HPLC variable using 0.1 M acetate buffer (pH 3.6) in the mobile phase for the mixture of Benzodiazepines (10 ppm each).

Boundary conditions:

Variable:	Upper	Lower	Step	Start
% CH_3CN	100	50	10	75
% Buffer	50	0	10	25
Flow rate	4.0	1.0	0.6	2.0

Experiment No.	% CH_3CN	% Buffer	Flow rate mil/min	CRF
1	75.00	25.00	2.00	3.48
2	84.66	15.34	2.16	4.75
3	77.59	22.41	2.58	5.27
4	87.25	12.75	2.74	4.70
5	84.19	15.81	2.55	4.94
6	77.12	22.89	2.98	5.74
7	73.34	26.66	3.39	6.65
8	66.75	33.26	3.41	8.02
9	58.02	41.98	3.85	11.86
10	71.64	28.36	3.10	6.34
11	68.66	31.34	3.36	7.57
12	53.34	46.66	3.82	12.98
13	62.17	37.83	3.59	8.80
14	58.93	41.07	3.71	10.51
15	52.44	47.56	3.95	13.16
16	55.46	44.54	3.86	12.26

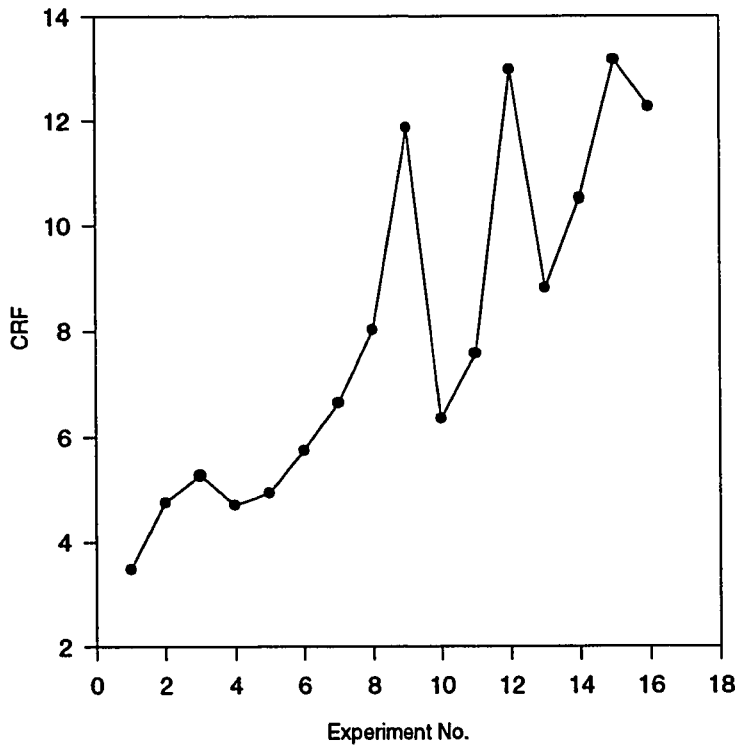


Figure 4.2.2. Simplex progress using 0.1 M acetate buffer with pH 3.6 in the mobile phase.

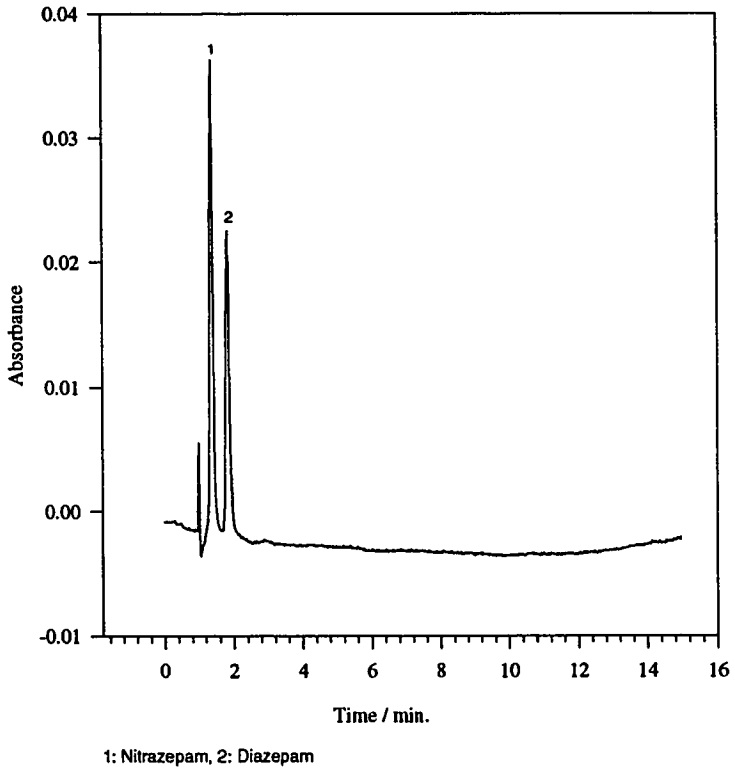
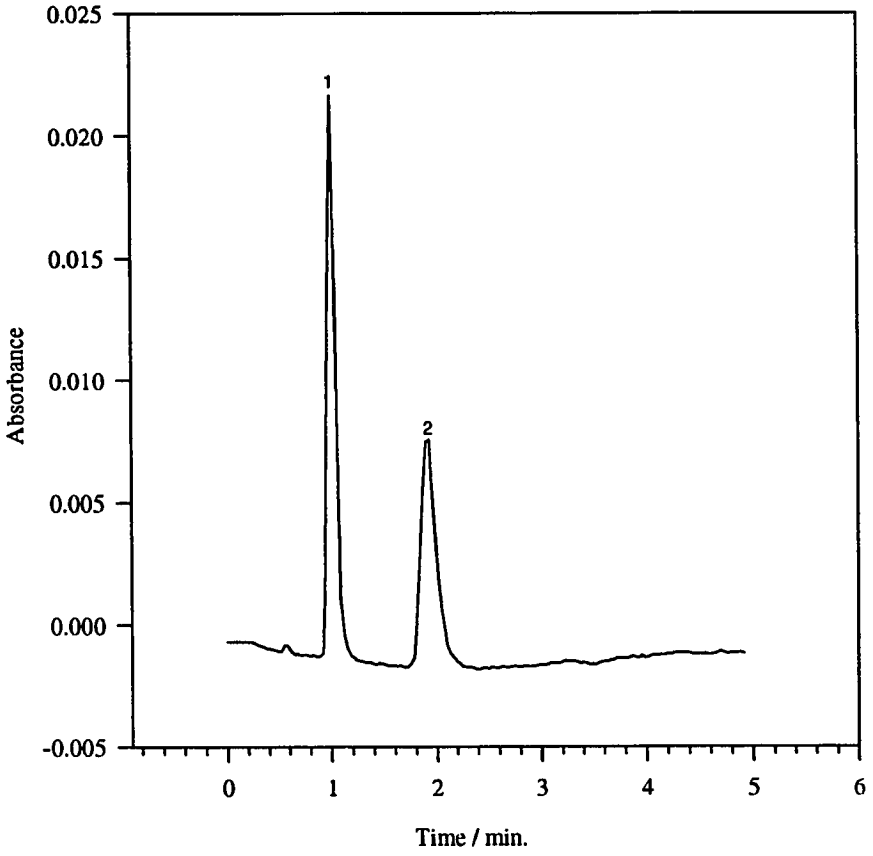
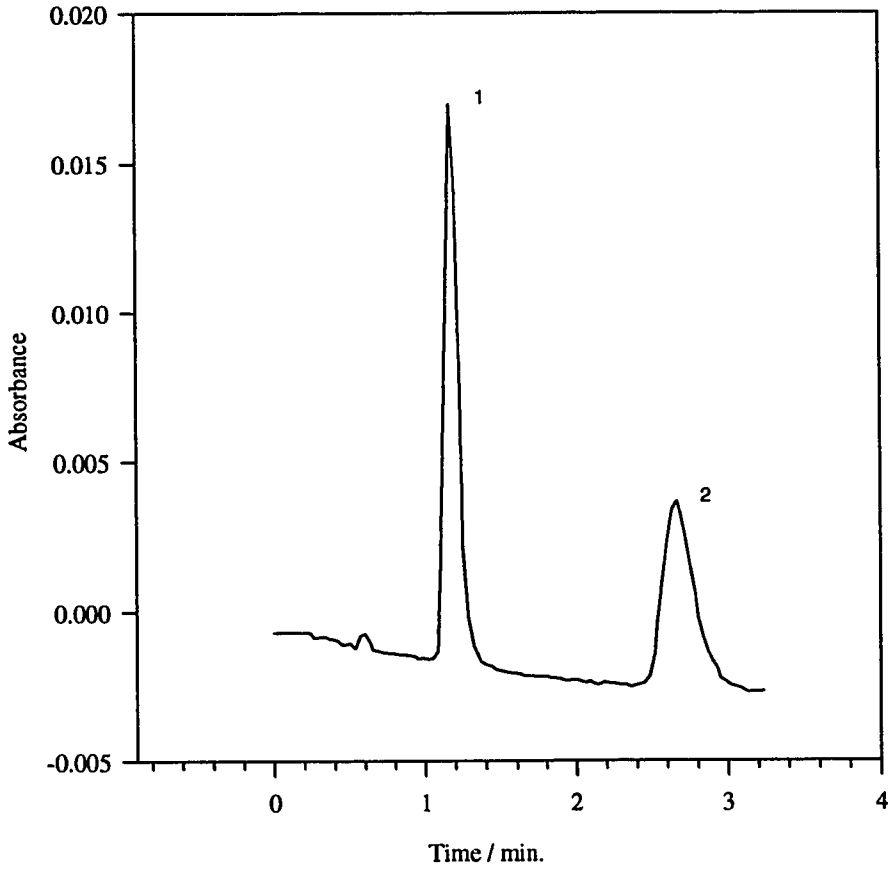


Figure 4.2.3a HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 2 in the simplex using 0.1 M acetate buffer at pH 3.6.



1: Nitrazepam, 2: Diazepam

Figure 4.2.3b HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 9 in the simplex using 0.1 M acetate buffer at pH 3.6.



1: Nitrazepam, 2: Diazepam

Figure 4.2.3c HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 15 in the simplex using 0.1 M acetate buffer at pH 3.6.

TABLE: 4.2.3 Boundary conditions and simplex optimization for HPLC variable using 0.1 M acetate buffer (pH 4.7) in the mobile phase for a mixture of Benzodiazepines.

Boundary conditions:

Variable:	Upper	Lower	Step	Start
% CH_3CN	95	75	4	80
% Buffer	25	5	4	20
Flow rate	4.0	1.5	0.5	2.5

Experiment No.	% CH_3CN	% Buffer	Flow rate mil/min	CRF
1	80.00	20.00	2.50	5.28
2	82.90	17.10	2.63	15.61
3	80.78	19.22	2.98	20.04
4	83.67	16.33	3.11	16.11
5	81.55	18.45	3.47	20.44
6	80.88	19.12	3.88	24.96
7	77.98	22.02	3.76	6.93
8	82.25	17.75	3.27	17.58
9	79.41	20.59	3.59	6.30
10	81.54	18.46	3.35	20.43
11	80.99	19.01	3.30	20.09
12	81.43	18.57	3.94	21.43
13	81.35	18.65	3.63	20.07
14	80.99	19.01	3.31	17.66
15	81.32	18.68	3.78	19.18
16	81.24	18.76	3.84	26.89
17	80.69	19.31	3.79	21.17
18	81.24	18.76	3.90	23.61
19	80.87	19.13	3.83	23.51
20	81.15	18.85	3.88	22.99

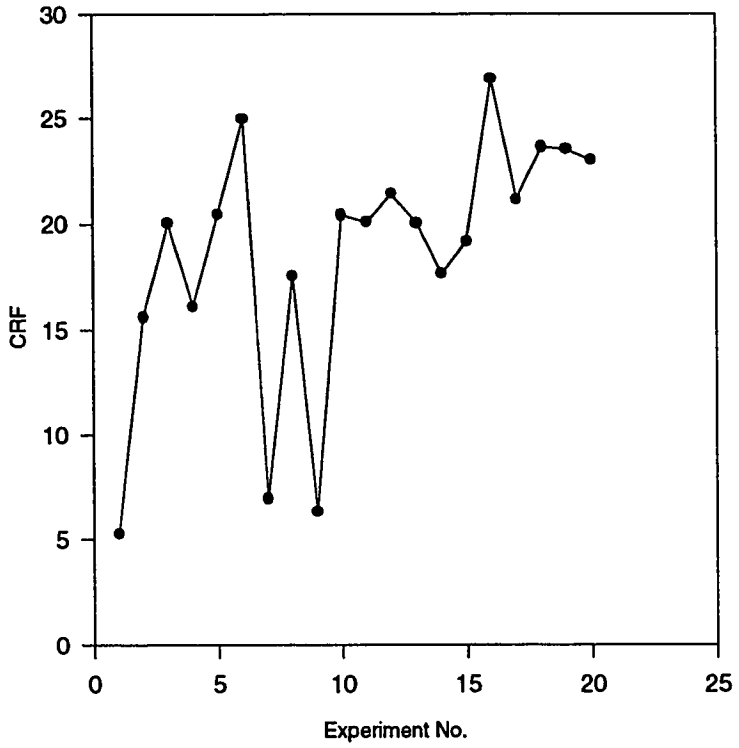
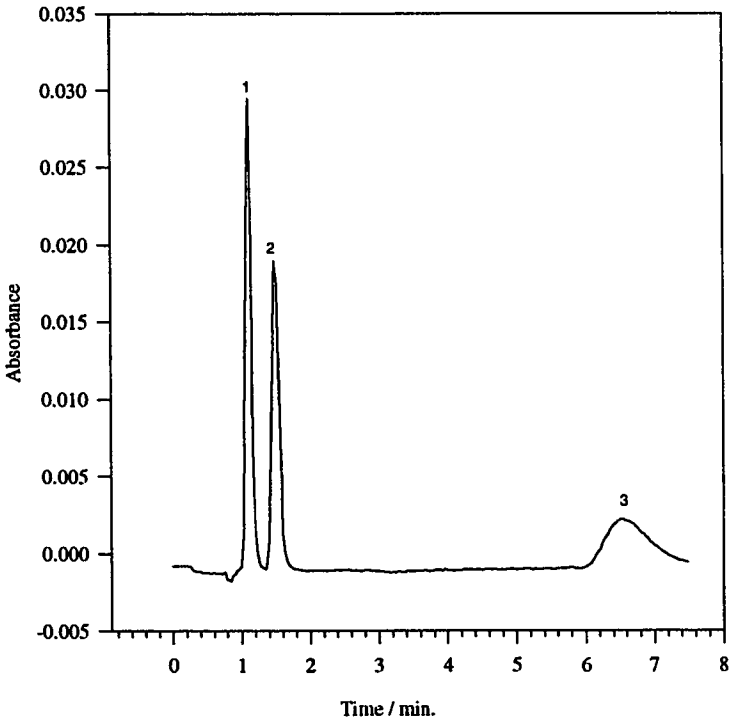
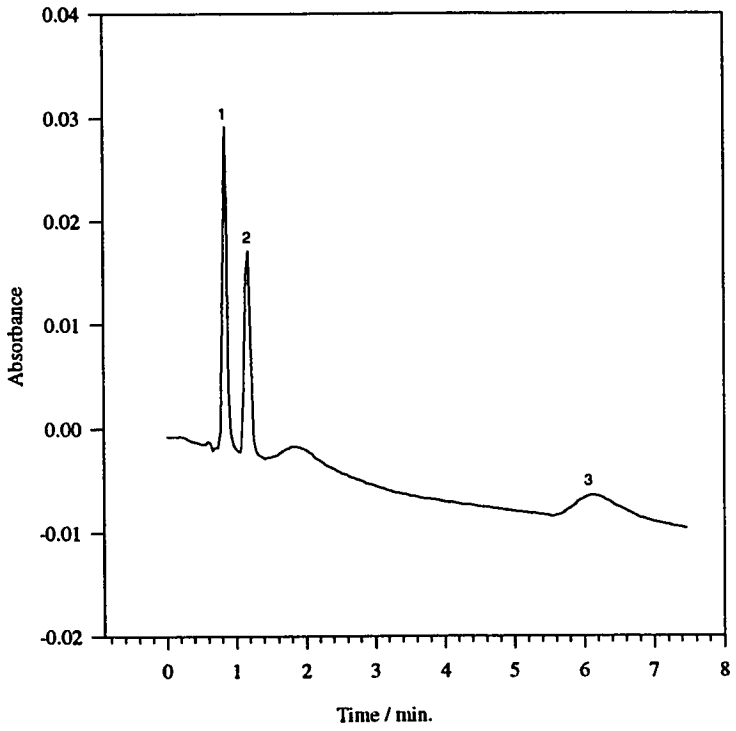


Figure 4.2.4 Simplex progress using 0.1 M acetate buffer with pH 4.7 in the mobile phase.



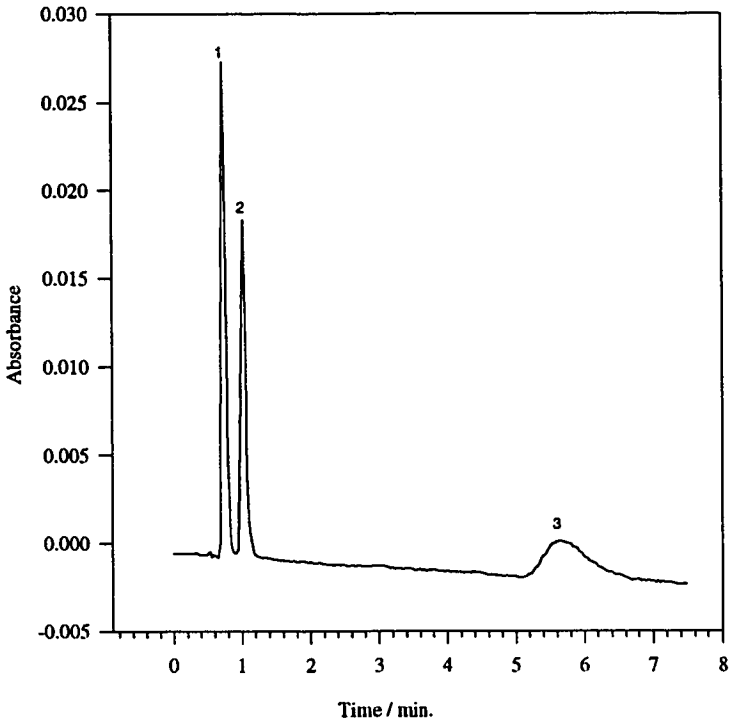
1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.2.5a HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 2 of the simplex using 0.1 M acetate buffer at pH 4.7.



1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.2.5b HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 14 of the simplex using 0.1 M acetate buffer at pH 4.7.



1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.2.5c HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 18 of the simplex using 0.1 M acetate buffer at pH 4.7.

TABLE: 4.2.4 Boundary conditions and simplex optimization for HPLC variable using 0.1 M acetate buffer (pH 6.0) in the mobile phase for the mixture of Benzodiazepines (10 ppm each).

Boundary conditions:

Variable:	Upper	Lower	Step	Start
% CH_3CN	100	70	6	80
% Buffer	30	0	6	20
Flow rate	4.0	1.0	0.6	2.0

Experiment No.	% CH_3CN	% Buffer	Flow rate mil/min	CRF
1	80.00	20.00	2.00	14.35
2	85.80	14.20	2.16	14.80
3	81.55	18.45	2.58	16.02
4	87.35	12.65	2.74	14.82
5	83.11	16.89	3.16	18.50
6	81.76	18.24	3.66	20.13
7	75.97	24.04	3.51	20.11
8	70.27	29.73	3.89	23.53
9	78.79	21.22	3.18	19.98
10	77.40	22.60	3.48	13.93
11	78.15	21.86	3.60	21.55
12	76.50	23.50	3.46	20.74
13	75.35	24.65	3.60	22.09
14	75.48	24.52	3.67	22.78
15	70.40	29.60	3.96	25.49
16	72.91	27.09	3.80	23.84
17	73.03	26.97	3.87	24.31
18	72.31	27.69	3.86	23.67
19	70.27	29.73	3.89	26.40
20	71.62	28.38	3.86	24.74

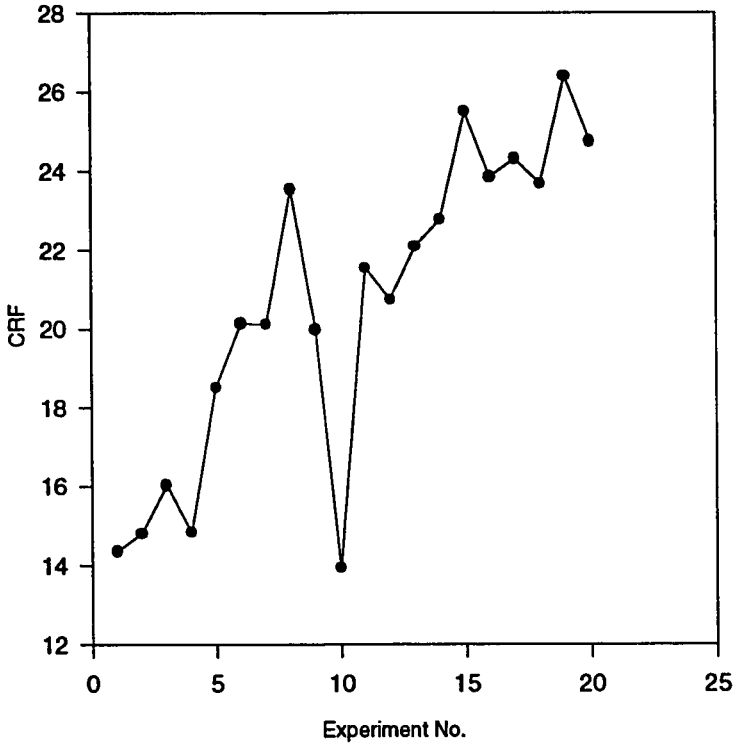
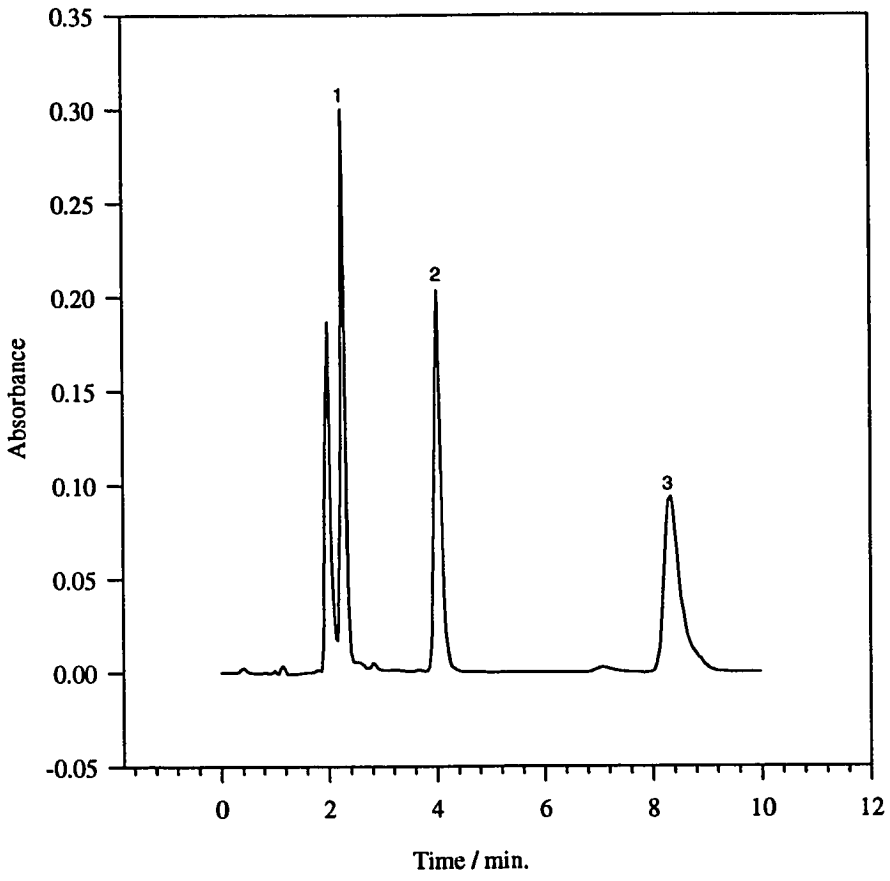
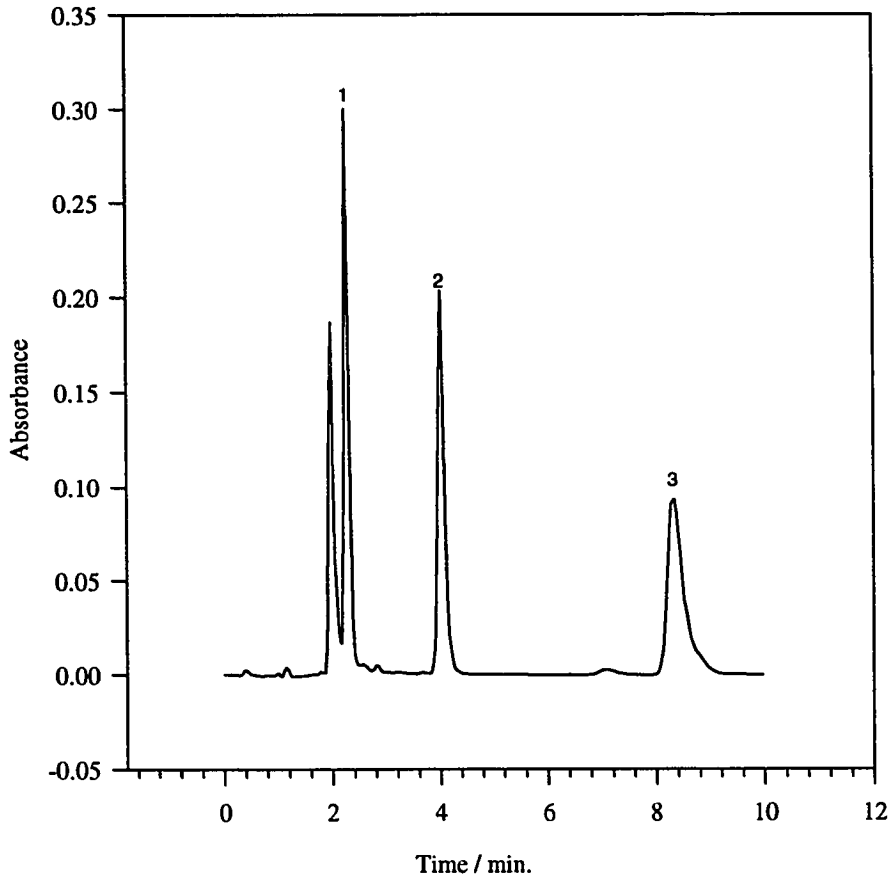


Figure 4.2.6 Simplex progress using 0.1 M acetate buffer with pH 6.0 in the mobile phase.



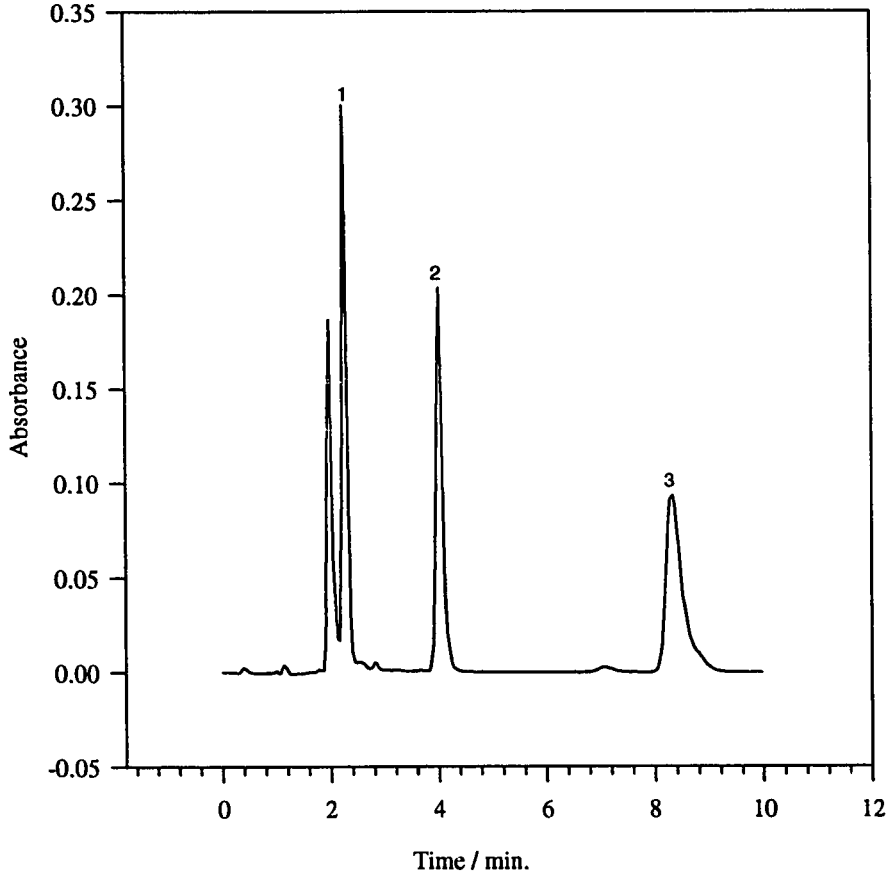
1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.2.7a HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 8 of the simplex using 0.1 M acetate buffer at pH 6.0.



1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.2.7b HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 10 of the simplex using 0.1 M acetate buffer at pH 6.0.



1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.2.7c HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 19 of the simplex using 0.1 M acetate buffer at pH 6.0.

4.3 Method development

Having determined the extent of the analysis required, the nature of the sample, the stationary phase and the mobile phase, the analyst is in a position to develop a method for the separation of a mixture of a Nitrazepam, Diazepam and Medazepam using simplex optimization procedure as a tool for determining the optimum conditions for the chemical and HPLC variables. In this task the variables to be optimized are the mobile phase composition, the flow rate, and the sample volume injected. Although the volume injected (*i.e.* sample concentration) is not a critical variable in the separation to be optimized, it has been introduced to see the loadability of the column. Table 4.3.1. lists the boundary conditions as well as the results obtained during the simplex progress. The initial simplex was adjusted on basis of the previous univariate optimization procedure. The results of the simplex progress are listed in table 4.3.1. The results show a significant correlation between the chemical and HPLC variables on one hand and the CRF and its parameters on the other hand. Figure 4.3.1. shows the variation of the CRF against the experiment number, which clearly indicates the movement of the simplex towards a global optima in a nice hill-climbing way, resulting in locating an optima at vertex number 21, after a search in a reflecting, expanding, and contracting fashion. The data obtained were further investigated, and figure 4.3.2. shows the effect of variation in the CRF as a factor of % CH_3CN in the mobile phase. It can be seen clearly that there is a general pattern, that as % CH_3CN in the mobile phase increases, the CRF increases. This can be attributed to the acetonitrile physical characteristics, being a weaker base and a much weaker acid than water, and which has a relatively high dielectric constant ($\varepsilon = 36.7$). The cumulative effect of these factors is that acetonitrile acts as a strongly differentiating solvent, as reflected by its small autoprotolysis constant ($pK_{HS} = 33.6$)[90]. This is why acetonitrile is one of the most important dipolar aprotic solvents ever used in HPLC.

TABLE: 4.2.5 Boundary conditions and simplex optimization for HPLC variable for method development for the mixture of Benzodiazepines (10 ppm each).

Boundary conditions:

Variable:	Upper	Lower	Step	Start
% CH_3CN	100	80	4	85
% Buffer	20	0	4	15
Flow rate	4.0	1.0	0.6	2.5
Concentration	500	10	98	100

Experiment No.	% CH_3CN	% Buffer	Flow rate mil/min	Conc. ng	CRF
1	85.00	15.00	2.50	100	14.03
2	87.83	12.17	2.62	111	12.94
3	85.71	14.29	2.97	111	15.51
4	85.71	14.29	2.62	145	13.42
5	83.11	16.89	2.78	126	14.20
6	83.51	16.49	2.88	80	15.13
7	83.22	16.78	3.25	112	11.34
8	84.56	15.45	2.69	103	14.13
9	83.66	16.34	3.06	109	15.09
10	85.47	14.53	3.17	74	15.55
11	86.65	13.35	3.36	47	17.15
12	86.91	13.09	3.08	50	15.48
13	89.34	10.66	3.40	59	18.97
14	87.55	12.45	3.41	95	17.68
15	86.78	13.22	3.18	89	16.49
16	87.31	12.69	3.29	78	16.72
17	88.38	11.62	3.50	56	20.35
18	87.54	12.46	3.40	59	18.24
19	89.29	10.71	3.45	21	19.41
20	90.47	9.53	3.50	32	24.09
21	89.36	10.64	3.44	48	24.64
22	89.34	10.66	3.47	33	22.25
23	91.07	8.94	3.44	19	23.37
24	91.25	8.75	3.45	33	21.20
25	89.82	10.18	3.46	33	21.02

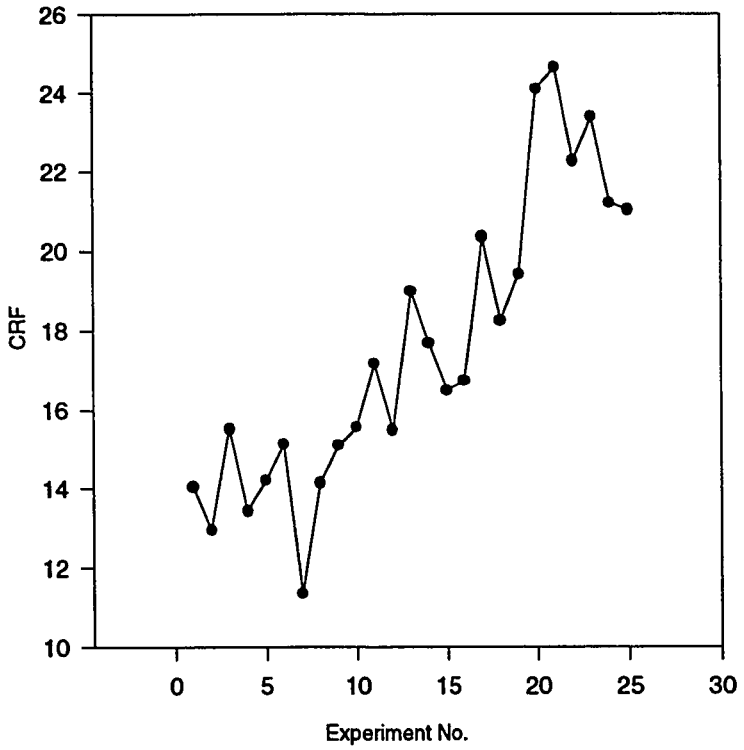


Figure 4.3.1 Simplex progress during method development.

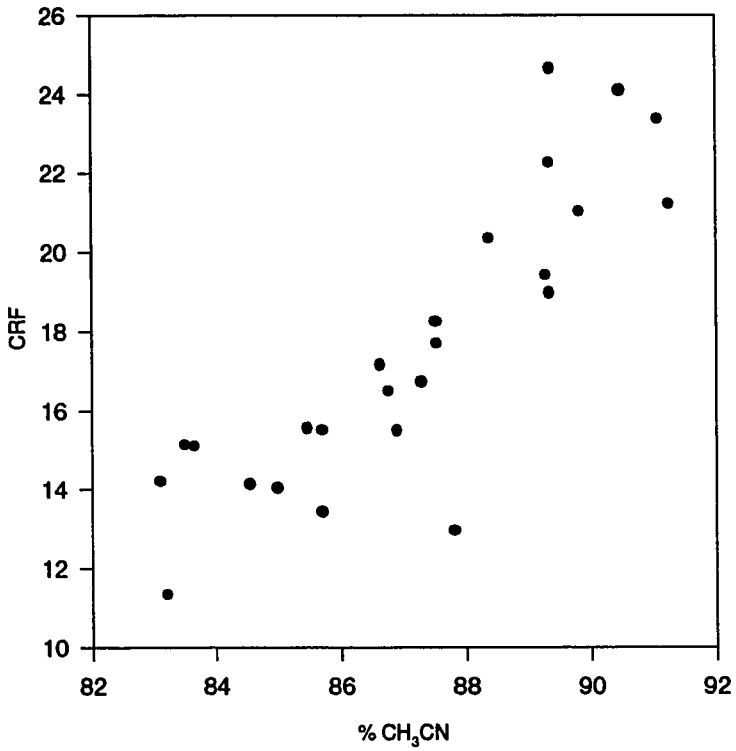


Figure 4.3.2 Effect of percentage CH_3CN in the mobile phase on CRF during simplex progress.

Figure 4.3.3. shows the effect of percentage of 0.1 M acetate buffer on the CRF. A pattern of decrease in the CRF as the aqueous acetate portion increases in the mobile phase, is mainly due to poor interaction of solute molecules with the aqueous mobile phase, since all the three benzodiazepines separated are practically insoluble in water. Also this is because a decrease in the % buffer means an increase in the % acteonitrile which causes an increase the CRF. The interactive effect of this binary mobile phase on the CRF was shown clearly on the three dimensional surface diagram, figure 4.3.4., from which one can visualize the movement of simplex toward the optima in a hill-climbing way as the combination of the mobile phase changes. This simplex leveling favors the increase in the % acetonitrile. Figure 4.3.5. shows the effect of the flow rate on the CRF. It is clear that the effect is minimal and over a very limited range after which it is insignificant. In fact flow rate has a more effect on the analysis time than on the resolution. As expected the effect of the concentration of solute on the CRF remained as according to theory *i.e.*, as concentration increased, the CRF decreased. This is in agreement with the theory, since as the sample size is sufficiently small, symmetrical Gaussian peaks can be obtained and hence a narrow peak width will result in the increase in the number of theoretical plates N leading to an increase in the resolution R_s. Figure 4.3.6. shows the trend of this relationship. Similar correlations were obtained for each individual HPLC and chemical parameter with each CRF variable *i.e.* N, k', α and R_s for each one of the three compounds in the mixture. This practice was done to see which CRF variable, if any, is affecting results more than the others. It is shown that the k' and α have the greatest effect over the plate number N.

The major difficulty encountered in HPLC analysis is the prediction of the retention time of each compound; it is a very complex phenomenon, and it is affected by the chromatographic variables as well as the solute chemical and physical characteristics. Many authors are trying to build a model or simulate an equation for

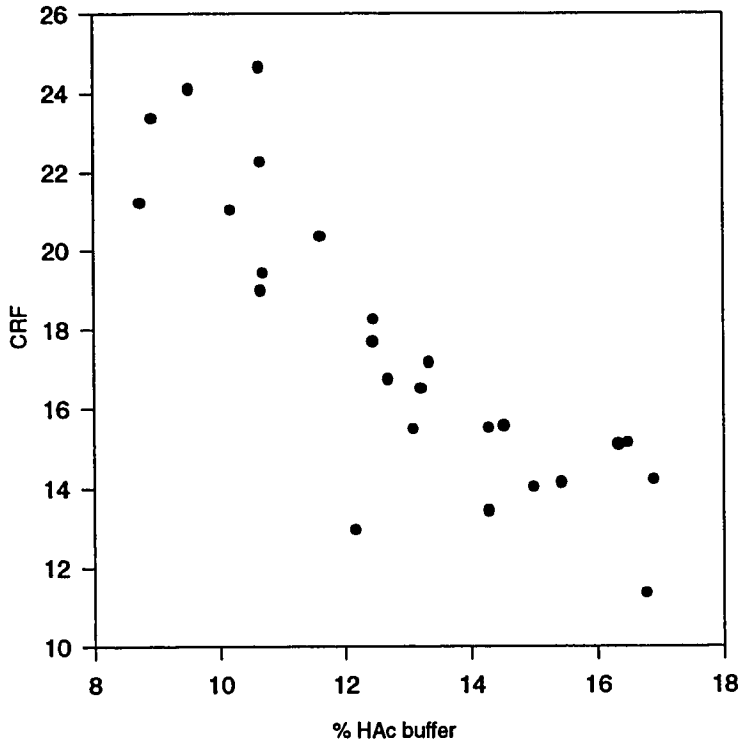


Figure 4.3.3 Effect of percentage 0.1 M acetate buffer (pH 6.0) on CRF during optimization.

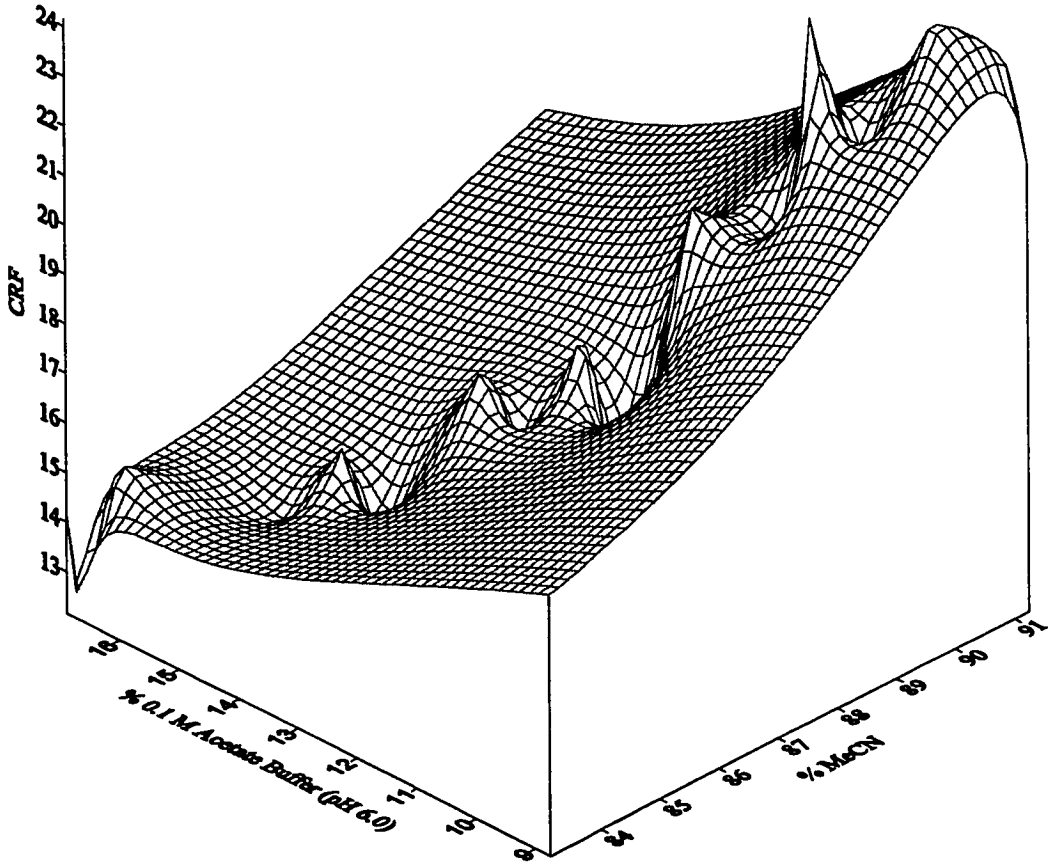


Figure 4.3.4 Surface diagram of the effect of the binary mobile phase on the CRF.

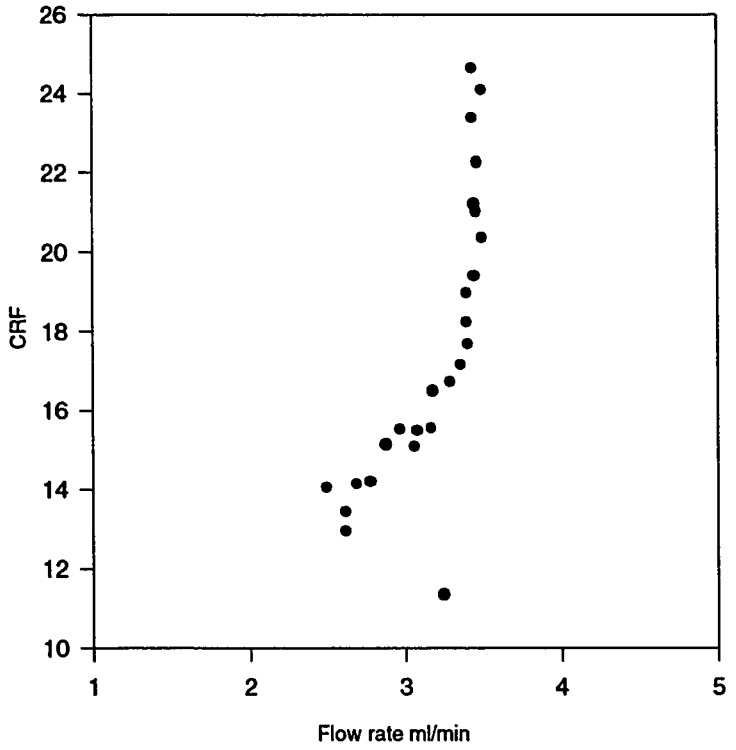


Figure 4.3.5 Effect of flow rate on CRF during simplex progress.

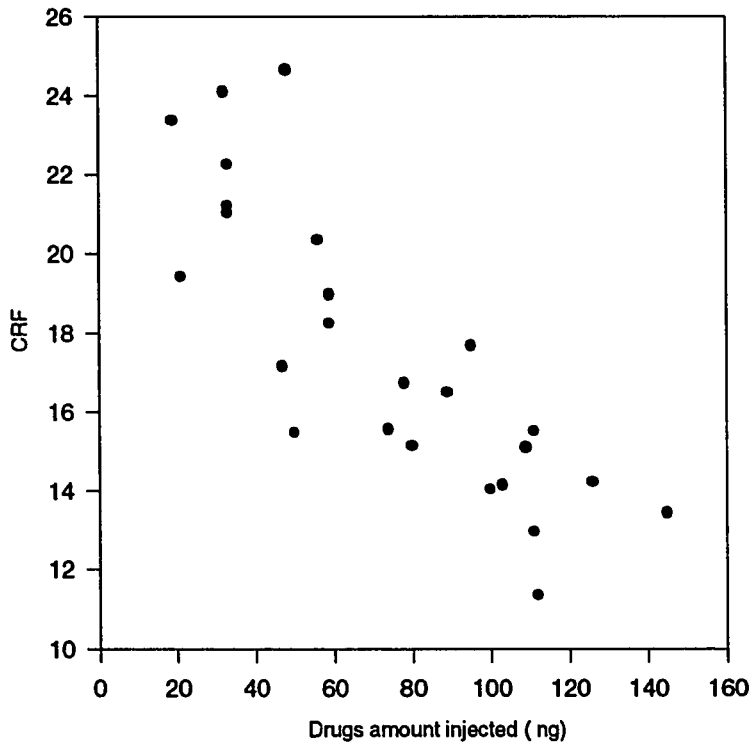
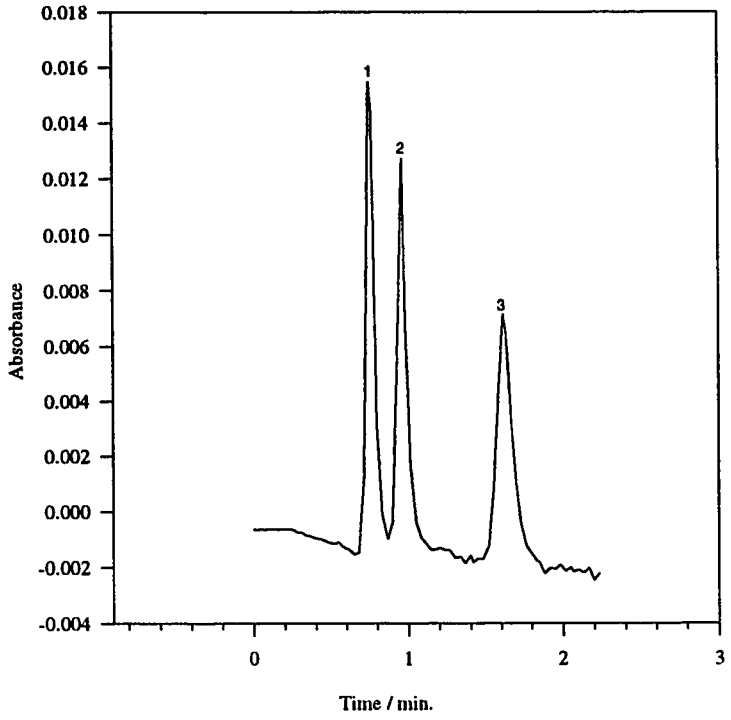


Figure 4.3.6 Effect of drugs injected concentration on CRF during optimization.

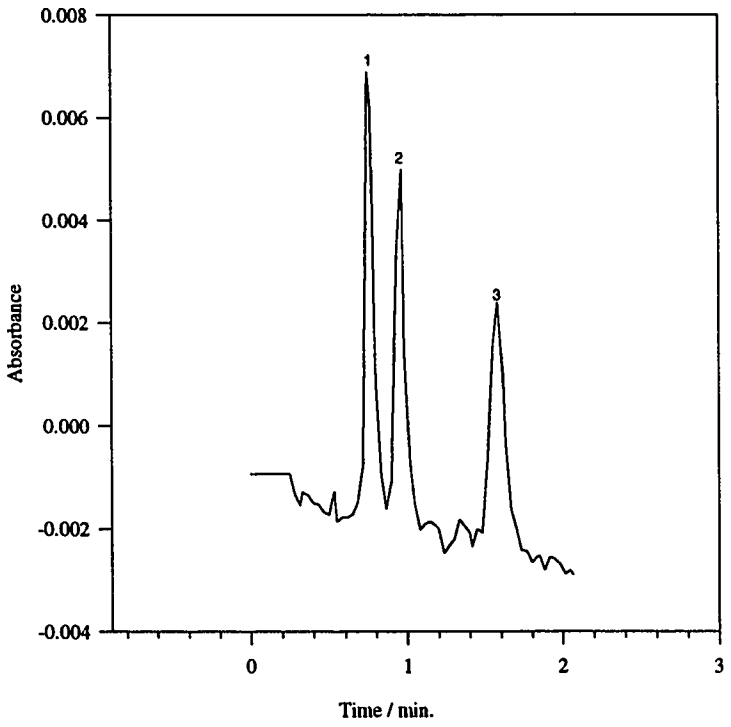
the prediction of the retention time of a specific system under a certain analytical and instrumental conditions[91-94]. Mechanism of retention of benzodiazepines in reverse phase high performance liquid chromatography in view of quantitative structure retention relationship was studied. It can be explained by the ability of a solute to participate in dispersive (London type) intermolecular interaction. These nonspecific, molecular size dependent interactions are obviously stronger between benzodiazepine solute molecule and a bulky hydrocarbonaceous moiety of Lichrosorb C18 stationary phase rather than between the same solute molecule and small molecules forming a mobile phase. The reverse holds true when considering parameters reflecting the ability of solute to take part in more structurally specific, size independent intermolecular interaction (so called polar interaction), like dipole-dipole, dipole-induced dipole, charge transfer and hydrogen bonding interactions. Such interactions, involving solute and polar molecules of eluent (acetonitrile, water, methanol), will increase with the magnitude of polarity. According to this mechanism, and after successful selection of the mobile and stationary phases, together with the help of the modified simplex optimization, it was feasible to separate the three compounds in the mixture in less than two minutes, which was predetermined at the beginning. Figures 4.3.7a, b, c, and d show different chromatograms obtained at different experimental variables around the optima during the simplex search.

From the above manifested results, a successful method for the separation of Nitrazepam, Diazepam and Medazepam has been developed. This method was represented by vertex no. 21 in the modified simplex search. The conditions of this developed method are, acetonitrile:acetate buffer, 89:11, flow rate 3.44 ml/min, volume injected 5 µl, ambient temperature and a Lichrosorb C18 column of 3.9 mm diameter and 20 cm length and a UV photo-diode array detection at 254 *n*m. These conditions were used in the analytical appraisal as well as the application.



1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.3.4.7a HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 17 during the simplex progress.



1: Nitrazepam, 2: Diazepam, 3: Medazepam

Figure 4.3.4.7b HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 20 during the simplex progress.

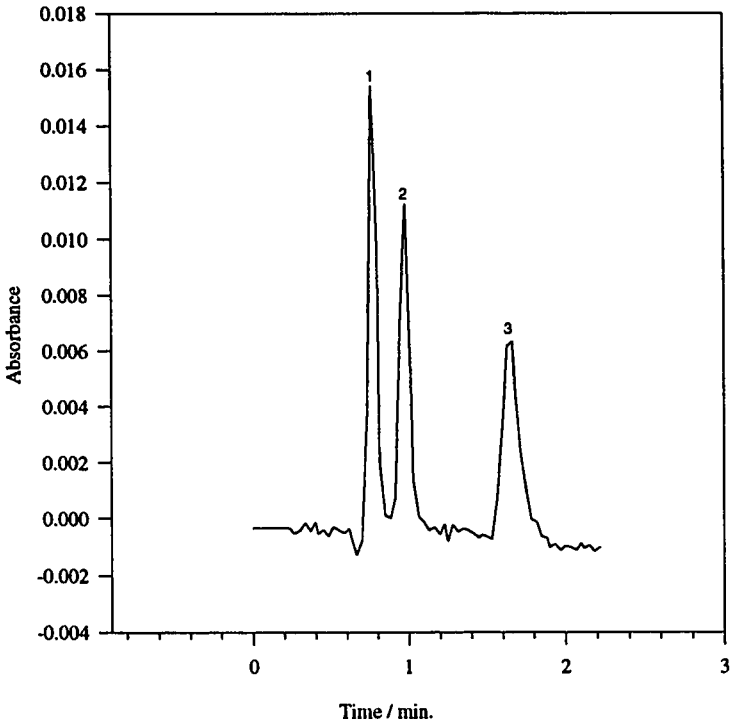


Figure 4.3.4.7c HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 21 during the simplex progress.

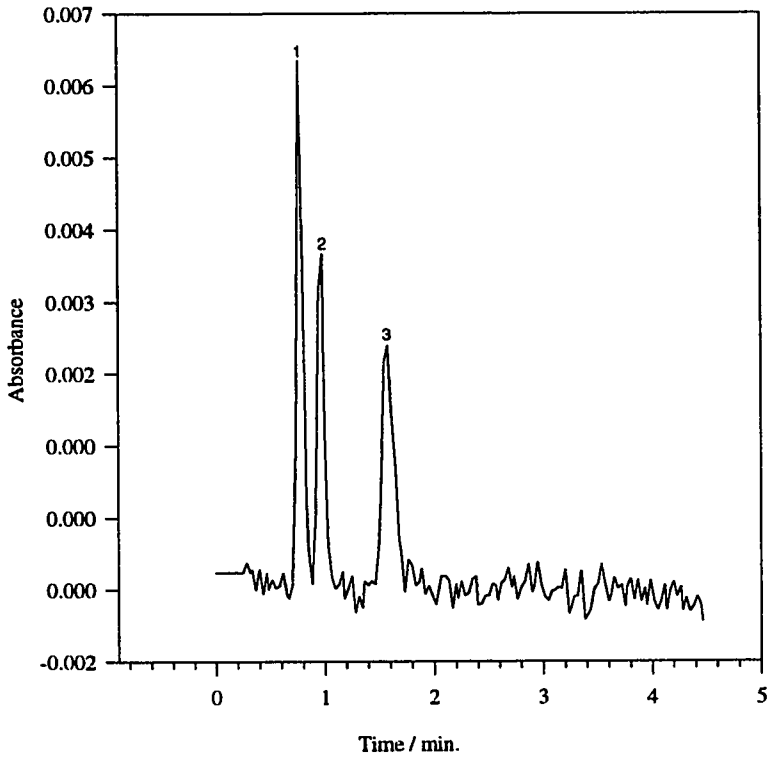


Figure 4.3.4.7d HPLC chromatogram obtained for the drug compounds under the experimental conditions of experiment No. 23 during the simplex progress.

4.4 Quantitative analysis

4.4.1 Introduction

Quantitation in HPLC is not different from any quantitative analytical method in the sense that good analytical practice is required. This means that due attention must be paid to the following points: (1) what type of analysis is required; is it a single or multicomponent analysis? (2) what are the concentration levels to be measured? (3) are pure standards available for calibration? (4) what interferences might be expected from the sample matrix? (5) what accuracy and precision is required?

For a multicomponent analysis it is essential that good resolution (preferably baseline Rs = 1.5) for all components is obtained. A knowledge of the concentration levels to be expected is also necessary since different levels have their own problems. For example, in trace analysis cross contamination of samples is a potential source of error. Noise and drift caused by solvent contaminants, flow variation and temperature fluctuation may decrease the precision. Purity determinations in the 95-100 % range are often difficult to produce. Pure standards are required for both qualitative and quantitative calibration of the detector. Detector response may vary markedly from analyte to analyte so that calibration is much more important than in other systems, such as GC. Accuracy of analysis refers to the nearness of the measured to the true value. This is dependent on calibrating the system with reliable standards and getting good resolution. The precision refers to the error on a series of replicate determinations. Reproducibility is the ability to obtain the same results from run to run, day to day, place to place, or even analyst to analyst. Thus precision is subject to systematic error and reproducibility to random error. Precise and accurate quantitative results are only obtained if due attention is paid to all phases of the analysis from first sampling to final results.

4.4.2 Measurement

The relative merits of peak height and peak area measurements have been fully understood[6]. In general peak area measurements should be used where control of mobile phase composition is poor, for distorted peaks and when operator and instrument variations other than flow rates are large. Precise flow control is necessary if peak area measurements are used. Peak height measurements should be used if flow rate control is poor and for trace components. Peak height measurements are more accurate than peak area measurements for overlapping peak. In this study, measurements of peak height and peak areas were done automatically by the digital and computing integrator of the system, since it gives the fastest and most accurate measurement, peak area was used in quantitation. Sometimes using manual integration within the integrator mode is required to determined the start, retention, and end time of each peak at the baseline and at the peak apex (height at maxima).

4.4.3 Calibration

Having obtained the raw data of peak heights or areas from the chromatogram or the integrator, this information must be converted into composition. For the purpose of accuracy, peak areas were used throughout this work, although peak heights are also available. Two methods were pursued: External standard and internal standard.

4.4.3.1 External standard method

Using the method developed, calibration plots are constructed by injecting automatically 5 µl of a standard mixture sample containing known concentrations of Nitrazepam, Diazepam and Medazepam which were adequate to cover the dynamic ranges of these

compounds in pharmaceutical as well as biological samples, i.e. 0.2-200 ppm. A plot of absorbance in terms of peak area versus weight or concentration for each compound is obtained. The plot should be linear and pass through the origin.

4.4.3.2 Internal standard method

This technique minimizes errors due to sample preparation, apparatus and technique. A known compound of fixed concentration is added to the unknown sample or standard to give a separate peak in the chromatogram. From the peak areas of the sample and the standard the composition may be determined. However the technique is useful when lengthy sample preparation is involved when recoveries may be variables. In this case the standard must be structurally similar to the sample. Calibration curves are obtained by chromatographing suitable volumes of calibration mixtures containing the compounds of the interest with a constant concentration of the internal standard. Peak areas of the compound of the interest are determined and the ratio peak area of compound (A_s)/peak area of internal standard (A_i) was plotted against the concentration of compound. Again the plot should be linear.

4.5 Determination of Nitrazepam in a mixture using external standard method

4.5.1 Introduction

Nitrazepam is 1,3-Dihydro-7-nitro-5-phenyl-2H-1,4-bezodiazepine-2-one, it is a white crystalline powder, with a molecular formula, $C_{15}H_{11}N_3O_3$, a molecular weight of 281.3 and having the structural formula shown in figure 4.5.1.1. Its melting point is 226-229 °C, it is insoluble in water, 0.8 g/100 ml in alcohol, 0.1 g/100 ml in diethyl ether. Nitrazepam is a tranquilizer, hypnotic, anti-depresive and sedative drug which is used for the treatment

of anxiety, conflict, worry, tension, feeling of oppression, sleep disturbance and status epileptics. It absorbs UV strongly at 254 nm.

4.5.2 Analytical appraisal

Using the developed method, Nitrazepam can be quantitated successfully. Bear's law was found to be valid in a wide range (0.2-200 ppm). Different standard solutions of Nitrazepam were prepared by appropriate dilution from the stock mixture, which have been described earlier in the experimental part. The standard solutions were injected in duplicate using the auto-injector of the system. The eluted peak of Nitrazepam which have been separated from Diazepam and Medazepam was monitored by the photodiode array detector at 254 nm. The peak area (area count) was determined by the system integrator using the manual integration mode. The results were tabulated in table 4.5.2.1. The measured peak absorbencies (in term of peak area) were plotted versus the concentration of Nitrazepam as shown in figure 4.5.2.1.

The straight line regression was obtained in the range of 0.2-200 ppm with a correlation coefficient of 0.9993 and intercept of 6.3557×10^{-5}. The regression equation is as follows:

$$A = 6.3557 \times 10^{-5} + 1.0656 \times 10^{-4} C$$

where

A = peak absorption (in term of peak area) of Nitrazepam at 254 nm.

C = Concentration of Nitrazepam in $\mu g/ml^{-1}$ (ppm)

This calibration curve and calibration equation was used later in the application for the determination of Nitrazepam in drug tablets.

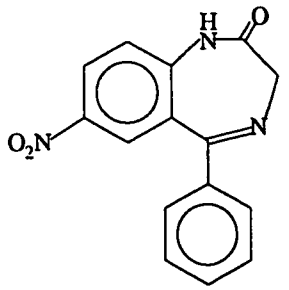


Figure 4.5.1.1 Chemical structure of Nitrazepam

TABLE 4.5.2.1 External standard calibration curve results obtained with the developed method for Nitrazepam.

Concentration ppm	Conc. Inj. ng.	Area-1 Au / min	Area-2 Au / min	Average Au / min
0.20	1.00	0.000028	0.000029	0.000029
1.00	5.00	0.000588	0.000597	0.000593
2.00	10.00	0.000294	0.000238	0.000266
5.00	25.00	0.000555	0.000612	0.000584
10.00	50.00	0.001075	0.001186	0.001131
15.00	75.00	0.001617	0.001610	0.001614
20.00	100.00	0.002243	0.002257	0.002250
30.00	150.00	0.003146	0.003166	0.003156
40.00	200.00	0.004359	0.004308	0.004334
50.00	250.00	0.005245	0.005029	0.005137
100.00	500.00	0.010746	0.010636	0.010691
150.00	750.00	0.015372	0.016225	0.015799
200.00	1000.00	0.021135	0.022165	0.021650

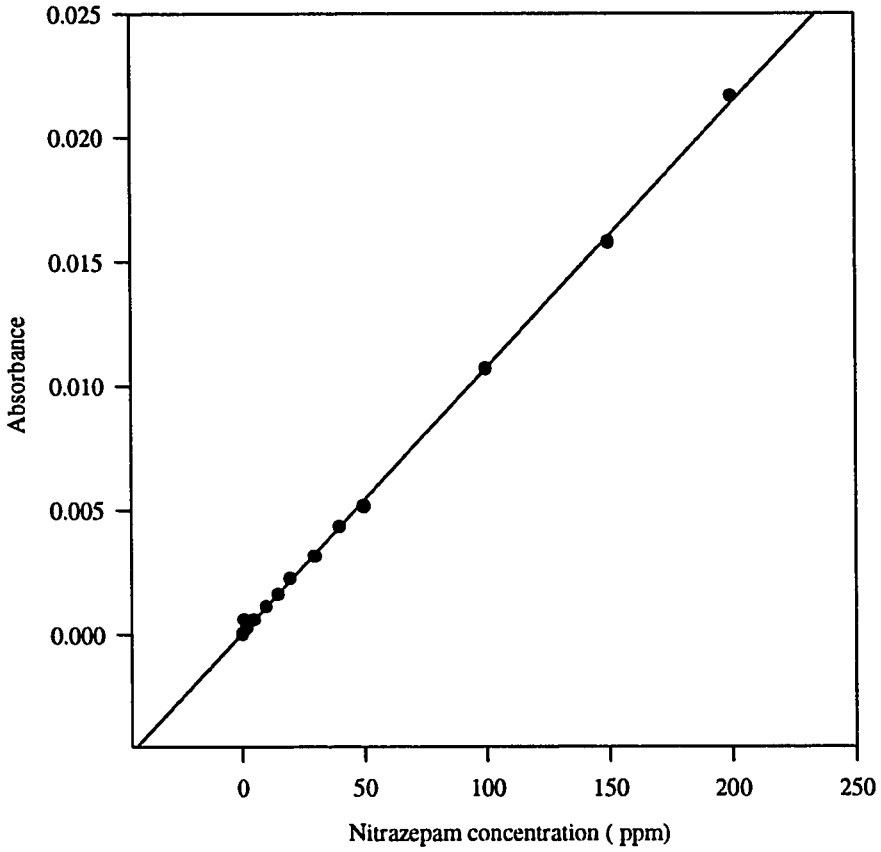


Figure 4.5.2.1 A typical calibration curve obtained for Nitrazepam using external standard method.

4.6 Determination of Diazepam in a mixture using external standard method

4.6.1 Introduction

Diazepam is 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one. It is an off-white to yellow, practically odorless, crystalline powder, with a molecular formula, $C_{16}H_{13}ClN_2O$, a molecular weight of 284.75 and having the structural formula shown in figure 4.6.1.1. Its melting point is 131-135 °C, its solubility in water is 0.05 mg/l, 32 mg/ ml in alcohol. Diazepam is a member of the group of benzodiazepine tranquilizers, which exert anxyiolytic, sedative, muscle relaxant, and anticonvalsant properties It is indicated for the symptomatic relief of anxiety, agitation and tension due to psycho neurotic states and transient situational disturbances. It absorbs UV at 254 nm.

4.6.2 Analytical appraisal

Again using the developed method, Diazepam can be quantitated successfully. Bear's law was found to be valid in a wide range (0.2-200 ppm). Different standard solutions of Diazepam were prepared by appropriate dilution from the stock mixture, which have been described earlier in the experimental part. The standard solutions were injected in duplicate using the auto-injector of the system. The eluted peak of Diazepam which has been separated from Nitrazepam and Medazepam was monitored by the photodiode array detector at 254 nm. The peak area (area count) was determined by the system integrator using the manual integration mode. The results were tabulated in table 4.6.2.1. The measured peak absorbencies (in terms of peak area) were plotted versus the concentration of Diazepam as shown in figure 4.6.2.1.

The straight line regression was obtained in the range of 0.2-200 ppm with a correlation coefficient of 0.9991 and intercept of 1.0082×10^{-5}. The regression equation is as follows:

$$A = 1.0082 \times 10^{-5} + 1.0238 \times 10^{-4} C$$

where

A = peak absorption (in term of peak area) of Diazepam at 254 nm.

C = Concentration of Nitrazepam in $\mu g/ml^{-1}$ (ppm)

This calibration curve and calibration equation was used later in the application for the determination of Diazepam in drug tablets.

4.7 Determination of Medazepam in a mixture using external standard method

4.7.1 Introduction

Medazepam is 7-chloro-2,3-dihydro-1-methyl-5-phenyl-1H-1,4-benzodiazepine. It is an off-yellow, practically odorless, crystalline powder, with a molecular formula, $C_{16}H_{15}N_2Cl$, a molecular weight of 270.77 and having the structural formula shown in figure 4.7.1.1. Its melting point is 95-97 °C, it is insoluble in water, 12 g/100 ml in alcohol. Medazepam is a member of the group of benzodiazepine tranquilizers, which exert anxyiolytic, sedative, short term action anticonvalsant. It is indicated for the symptomatic relief of anxiety, and sleep disturbances. It absorbs UV at 254 nm.

4.7.2 Analytical appraisal

As before, with the help of the developed method, Medazepam can be quantified

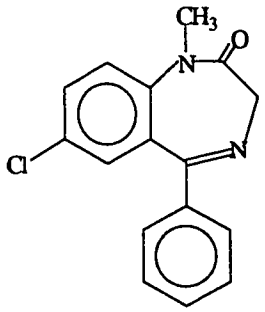


Figure 4.6.1.1 Chemical structure of Diazepam

TABLE 4.6.2.1 . External standard calibration curve results obtained with the developed method for Diazepam

Concentration ppm	Conc. Inj. ng.	Area-1 Au / min	Area-2 Au / min	Average Au / min
0.20	1.00	0.000015	0.000022	0.000019
1.00	5.00	0.000564	0.000521	0.000543
2.00	10.00	0.000262	0.000303	0.000283
5.00	25.00	0.000503	0.000540	0.000522
10.00	50.00	0.001105	0.001041	0.001073
15.00	75.00	0.001613	0.001562	0.001588
20.00	100.00	0.001928	0.002074	0.002001
30.00	150.00	0.002960	0.002981	0.002971
40.00	200.00	0.004155	0.003994	0.004075
50.00	250.00	0.004832	0.004764	0.004798
100.00	500.00	0.009529	0.010494	0.010012
150.00	750.00	0.015269	0.015261	0.015265
200.00	1000.00	0.019365	0.022200	0.020783

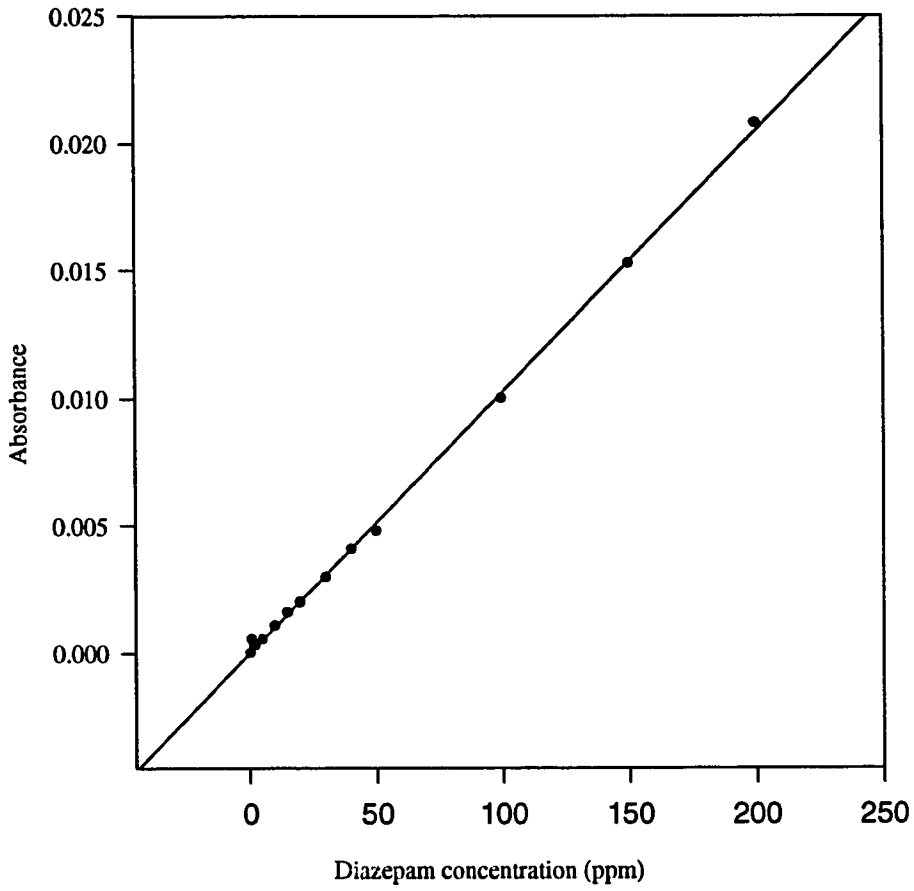


Figure 4.6.2.1 A typical calibration curve obtained for Diazepam using external standard method.

successfully. Bear's law was found to be valid in a wide range (0.2-200 ppm). Different standard solutions of Medazepam were prepared by appropriate dilution from the stock mixture, which have been described earlier in the experimental part. The standard solutions were injected in duplicate using the auto-injector of the system.

The eluted peak of Diazepam which have been separated from Nitrazepam and Diazepam was monitored by the photodiode array detector at 254 nm. The peak area (area count) was determined by the system integrator using the manual integration mode. The results were tabulated in table 4.7.2.1. The measured peak absorbencies (in terms of peak area) were plotted versus the concentration of Medazepam as shown in figure 4.7.2.1.
The straight line regression was obtained in the range of 0.2-200 ppm with a correlation coefficient of 0.9989 and intercept of 1.4295×10^{-5}. The regression equation is as follows:

$$A = 1.4295 \times 10^{-5} + 1.0888 \times 10^{-4} C$$

where

A = peak absorption (in term of peak area) of Medazepam at 254 nm.

C = Concentration of Medazepam in $\mu g/ml^{-1}$ (ppm)

4.8 Determination of Nitrazepam and Medazepam in a mixture using Diazepam as internal standard

Since any member of the benzodiazepine family can be used as internal standard for the determination of other benzodiazepine in a mixture, without any elution order problems, diazepam was used as internal standard for the determination of Nitrazepam and medazepam in a mixture. It is not the customary practice to use the analyte as internal

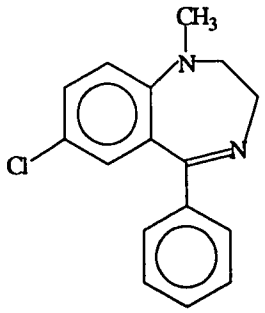


Figure 4.7.1.1 Chemical structure of Medazepam

TABLE 4.7.2.1 . External standard calibration curve results obtained with the developed method for Medazepam

Concentration ppm	Conc. Inj. ng.	Area-1 Au / min	Area-2 Au / min	Average Au / min
0.20	1.00	0.000017	0.000054	0.000036
1.00	5.00	0.000605	0.000500	0.000553
2.00	10.00	0.000241	0.000228	0.000235
5.00	25.00	0.000582	0.000594	0.000588
10.00	50.00	0.001186	0.001190	0.001188
15.00	75.00	0.001700	0.001682	0.001691
20.00	100.00	0.001989	0.002232	0.002111
30.00	150.00	0.003155	0.002993	0.003074
40.00	200.00	0.004426	0.004413	0.004420
50.00	250.00	0.005412	0.005087	0.005250
100.00	500.00	0.010301	0.010606	0.010454
150.00	750.00	0.016640	0.015885	0.016263
200.00	1000.00	0.021623	0.022651	0.022137

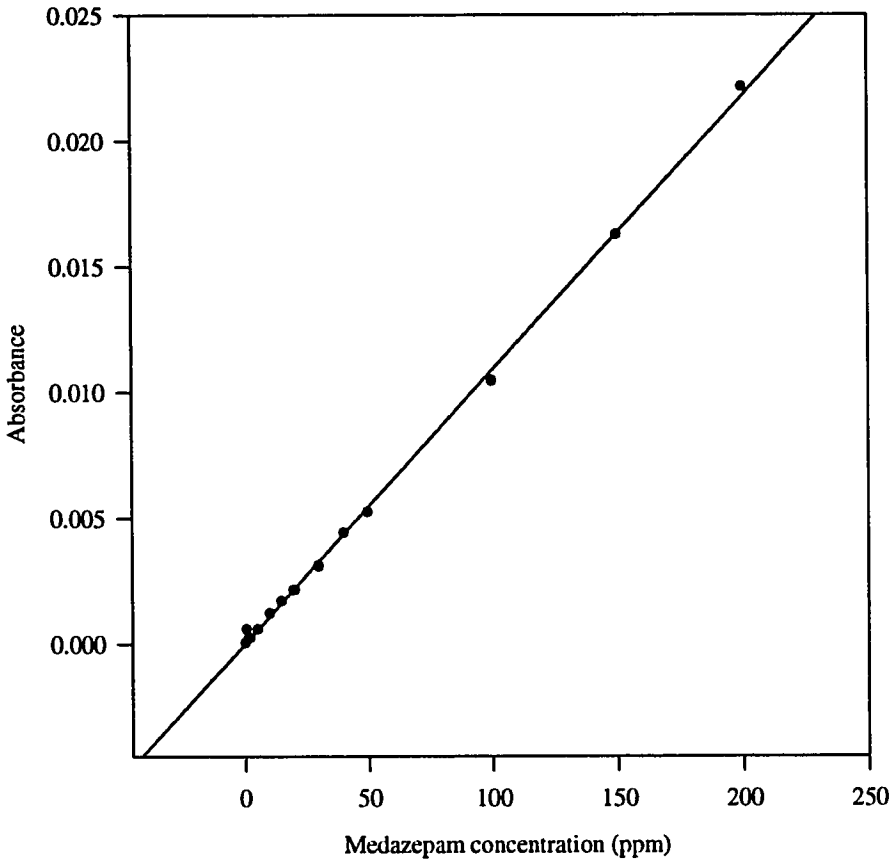


Figure 4.7.2.1 A typical calibration curve obtained for Medazepam using external standard method.

standard but this was included here as a trial. A fixed concentration of 20 ppm of diazepam (equivalent to 100 ng absolute weight injected in the column) was added to a series of concentrations of standard mixture containing nitrazepam and medazepam, this series representing a dynamic range of 1-100 ppm, which covers the pharmaceutical and the biological ranges. Injecting these concentrations in duplicate under the instrumental conditions specified by the method developed, then after obtaining and integrating the chromatograms, it gives the data listed in tables 4.8.1.1. and 4.8.1.2. for nitrazepam and medazepam respectively. The ratio of the areas of the sample and the internal standard for nitrazepam and medazepam were plotted versus the sample concentrations in the mixture, these plots were shown in figures 4.8.1.1 and 4.8.1.2 respectively. The nitrazepam plot gives a regression line with intercept of 0.01087, slope of 0.01027 and regression coefficient (r^2) of 0.999. A concentration of an unknown sample could be obtained from the following equation

$$\frac{A_s}{A_i} = 0.01087 + 0.01027C$$

where

A_s = area of the sample

A_i = area of the internal standard

C = Concentration of Nitrazepam in µg/ml (ppm).

The Medazepam plot gives a regression line with intercept of 0.01019, slope of 4.2512 x 10^{-3} and regression coefficient (r^2) of 0.9993. A concentration of an unknown sample could be obtained from the following equation

$$\frac{A_s}{A_i} = 0.01019 + 4.2512x10^{-3}C$$

where

A_s = area of the sample

TABLE 4.8.1.1 Internal standard calibration data obtained from the chromatogram of Nitrazepam using Diazepam as internal standard.

		Nitrazepam			Diazepam			
Conc. ppm	Inj.wt. ng.	Area1 Au*m	Area2 Au*m	Average	Area1 Au*m	Area2 Au*m	Average	As/Ai
1.00	5.00	0.000146	0.000206	0.000176	0.002064	0.002093	0.002079	0.084676
2.00	10.00	0.000383	0.000230	0.000307	0.002237	0.002217	0.002227	0.137629
5.00	25.00	0.000567	0.000592	0.000580	0.002214	0.002108	0.002161	0.268163
10.00	50.00	0.001072	0.001098	0.001085	0.002162	0.002117	0.002140	0.507128
15.00	75.00	0.001670	0.001675	0.001673	0.002078	0.002073	0.002076	0.805830
20.00	100.00	0.002106	0.002198	0.002152	0.002094	0.002176	0.002135	1.007963
30.00	150.00	0.003228	0.003247	0.003238	0.002178	0.002148	0.002163	1.496764
40.00	200.00	0.004264	0.004339	0.004302	0.001897	0.002098	0.001998	2.153442
50.00	250.00	0.005199	0.005137	0.005168	0.002077	0.002067	0.002072	2.494208
100.00	500.00	0.010246	0.010488	0.010367	0.002003	0.002005	0.002004	5.173154

TABLE 4.8.1.2 Internal standard calibration data obtained from the chromatogram of Medazepam using Diazepam as internal standard.

		Medazepam			Diazepam			
Conc. ppm	Inj. ng.	Area1 Au*m	Area2 Au*m	Average	Area1 Au*m	Area2 Au*m	Average	As/Ai
1.00	5.00	0.000113	0.000169	0.000141	0.002064	0.002093	0.002079	0.067837
2.00	10.00	0.000224	0.000206	0.000215	0.002237	0.002217	0.002227	0.096542
5.00	25.00	0.000491	0.000469	0.00048	0.002214	0.002108	0.002161	0.222119
10.00	50.00	0.001122	0.00131	0.001216	0.002162	0.002117	0.00214	0.568357
15.00	75.00	0.001708	0.001723	0.001716	0.002078	0.002073	0.002076	0.826548
20.00	100.00	0.002013	0.002194	0.002104	0.002094	0.002176	0.002135	0.985246
30.00	150.00	0.003289	0.003073	0.003181	0.002178	0.002148	0.002163	1.470643
40.00	200.00	0.004243	0.004072	0.004158	0.001897	0.002098	0.001998	2.081352
50.00	250.00	0.005443	0.005005	0.005224	0.002077	0.002067	0.002072	2.521236
100.00	500.00	0.010454	0.010043	0.010249	0.002003	0.002005	0.002004	5.114022

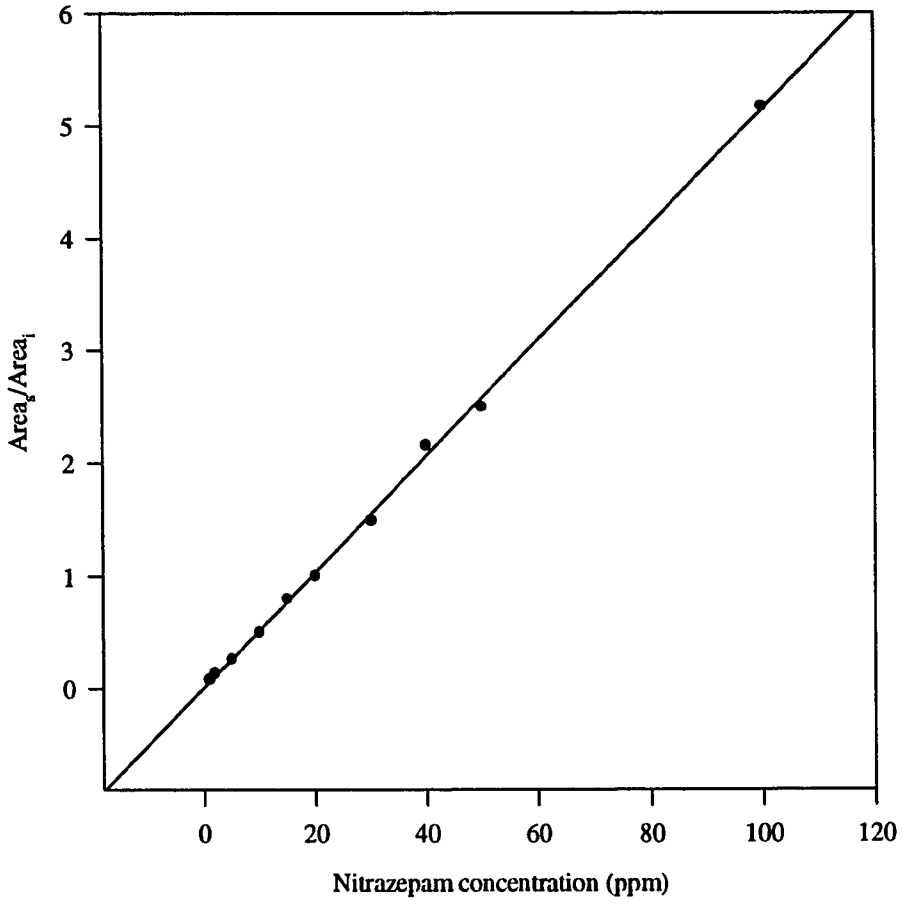


Figure 4.8.1.1 A typical calibration curve for Nitrazepam using Diazepam as internal standard.

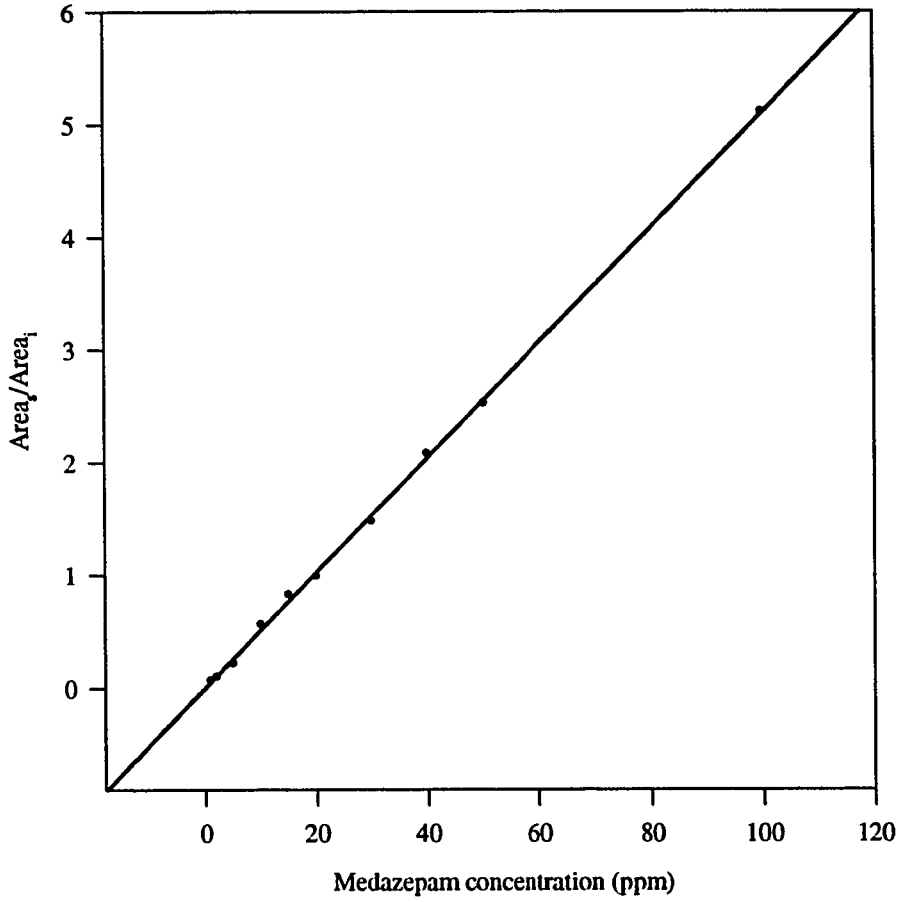


Figure 4.8.1.2 A typical calibration curve for Medazepam using Diazepam as internal standard.

A_i = area of the internal standard

C = Concentration of Medazepam in μg/ml (ppm)

4.9 Applications

Establishment of the validity of the developed method was carried out by analyzing the proprietary drugs containing Nitrazepam and Diazepam, which are the only two available drugs from local dispensaries. First the two drugs were measured based on the external standard calibration method, and later, using the diazepam as internal standard, nitrazepam was measured by the internal standard calibration method. A content of ≥5 tablets of each drug were weighed, crushed and powdered carefully. A weighed portion of powder, equivalent to 5 mg nitrazepam and 10 mg diazepam was accurately transferred to a 100 volumetric flask, then ≈ 40 ml of mobile phase of acetonitrile:acetate buffer, 89:11 was added, sonicated while heating for 30 minutes, filtered through 0.45 μm filter, the filtrate was washed several times with warmed mobile phase, the volume was completed to 100 ml to give a concentration of 50 ppm Nitrazepam and 100 ppm Diazepam, further dilution was made to give a concentration of 5 and 10 ppm respectively. Also a mixture containing 5 ppm Nitrazepam and 10 ppm Diazepam was prepared for the determination of nitrazepam using diazepam as internal standard. Using the method developed the solutions were injected using the auto sampler and chromtogramed at 254 nm, the results of this study were listed in table 4.9.1. for the external standard and table 4.9.2. for the internal standard.

From the results the internal standard method is more accurate than the external. Both methods shows good recoveries. For the first instance the recovery was not as reported, that is mainly due to extraction procedure, when the drug sample was dissolved in acetonitrile only with mechanical shaking, then filtering through Wattman 42. Later the

TABLE 4.9.1 Results obtained with the developed method for the determination of nitrazepam(5 mg) and diazepam (10 mg) in tablets using the external standard method.

Drug™	Active ingredient	supplier	Area Au*min	Found mg	present mg	Recovery %	Error %
Mogadon	Nitrazepam*	Roche	0.000601	5.07	5.07	101.4	1.4
Valume	Diazepam**	Roche	0.001049	10.16	10.16	101.6	1.6

* Standard deviation (n = 5) = ±0.014

** Standard deviation (n = 5) = ±0.110

TABLE 4.9.2 Results obtained with the developed method for the determination of nitrazepam(5 mg) using diazepam (10 ppm) as internal standard.

Drug™	Active ingredient	supplier	$\frac{A_s}{A_i}$	Found mg	Claimed mg	Recovery %	Error %
Mogadon	Nitrazepam*	Roche	0.5312	5.07	5.07	101.4	1.4

* Standard deviation (n = 5) = ±0.194

TABLE 4.9.3 Comparison of the HPLC developed method with the official standard method for Diazepam in tablets[95].

Parameter	Official method	Developed method
HPLC system	Tracor 950 solv. pump	Waters 600E solv. pump
Column	C18 μBondapack, 10 μm stainless steel, 30 cm x 3.9 mm id (Waters associates, Inc.)	C18 Lichrosorb, 5 μm stainless steel, 20 cm x 3.9 mm id (Hewllet Packared.)
Mobile phase	MeOH:Water, 65:35	MeCN:acetate buffer, 89:11
Flow rate	1.2 ml/min	3.44 ml/min
Temperature	Ambient	Ambient
Injection system, vol. Inj.	Auto-manual, 10-20 μl	Autoinjction, 5 μl
Detector & wavelength	Tracor 970A UV, 254 nm	Photodiode array 991, 254 nm
Detector sensitivity	60-90% AUFS	80-90% AUFS
Diazepam standard, Solv.	USP Ref. Std., MeOH	Roche generic std., MeCN
Diazepam tablets	Unknown	Valume, Roche, Switzerland
Calibration method	Internal Std., *p*-tolualdehyde	External Std.
Diazepam retention time	> 8.0 min	< 1.0 min
Detection limit	Unknown	200 ppb
Recovery %	103.2 %	101.6 %
Accuracy	±0.20 ppm	±0.11 ppm

extraction method was modified to include heating, sonication and filtering through Millipore 0.45 μm and further through Millipore 0.22 μm.

From the drugs assayed in this work, there has been only one liquid chromatographic method (for quantitative determination of diazepam in tablets) which has been adopted by official final action[95]. There is no official method for nitrazepam and medazepam. Table 4.9.3. lists a comparison between the official method and the developed method for the determination of diazepam in tablets.

4.10 Conclusion

The method developed is accurate, sensitive and relatively fast with respect to high performance liquid chromatographic methods. It is quite suitable for the assay of Nitrazepam, Diazepam and Medazepam in drug tablets in a mixture or as single component, without suffering interferences from excipients and other tablets additives. Also this gives the possibility for the application of the method on other benzodiazepines family members safely. The successful optimization of the chromatographic and chemical conditions using a modified simplex algorithm, gives a good separation between the three compound in a very reasonable requested time. This advantage makes the method suitable for routine laboratory analysis, and safe time in research and development works. Compared with the official method reported for Diazepam (only), this method is faster, accurate, more sensitive and consume fewer reagents and chemicals. However, the relatively higher flow rate (3.44 ml/min) which may cause sometimes a band broadening, which leads to tailing and peaks overlapping, and the high sophistication of the photodiode array detection system, may slow the application of this method on a large scale in drugs industries or in clinical laboratories.

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