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ASSAY DEVELOPMENT IN STABILITY TEST METHODS

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ABSTRACT

Stability-indicating analytical methods are developed to monitor the stability of pharmaceutical dosage forms during the investigational phase of drug development, and, once the drug is marketed, for the ongoing stability studies which must be conducted. The development of these methods for pharmaceutical dosage forms forms can be approached from several avenues. Methods can be developed which measure the amount of drug remaining, the amount of drug lost (or the appearance of degradation products), or both.

Traditionally, the analytical methods used to monitor the stability of dosage forms have involved a generally non-specific spectrophotometric or titrimetric procedure for the assay of the active coupled with thin layer chromatography for the estimation of impurities and degradation products. In the last five years, this

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approach has changed dramatically. Currently, the method of choice for the quantitation of the active and degradation products is rapidly becoming high performance liquid chromatography. This method has obvious advantages since it both separates and measures and it lends itself well to automation. The disadvantages are that, in the absence of automation, the technique can be time-consuming, it is by no means universal, and it is relatively expensive. Recent advances in column technology have reduced some separation times to seconds and, in the next few years, this technique may find even greater utility.

HPLC, however, is not the only way to go. Other chromatographic methods still find a place, particularly gas chromatography when the stability of the component of interest does not pose a problem and thin layer chromatography for the rapid determination of degradation products. Other methods may also be used, including electrometric, e.g., polarography, and spectrophotometric, e.g., fluorimetry or NMR. The choice of an appropriate method must depend on both a scientific and practical evaluation of the drug and its dosage form.

Once an analytical method is chosen, the most important aspect of the development of a stability-indicating procedure is method validation. Validation should include evaluation of the following parameters: specificity, linearity, precision, accuracy, sensitivity, and ruggedness.

There are many other aspects to stability that could also be considered, e.g., the stability of the bulk drug and physical and organoleptic changes in a dosage form. These should be part of a

separate discussion. It very often happens that, during the course of product development, analytical methods evolve. As more is learned about the drug and its dosage form, methods can be refined and revised.

INTRODUCTION

Developing methods for the determination of the stability of pharmaceutical dosage forms for both IND and post-NDA studies can be a time-consuming activity. The amount of effort expended depends upon the nature of the drug, the complexities of the dosage forms, and the amount of information already available. This activity may require input from a number of groups in a corporation in order to develop well-validated procedures in the most efficient manner. Coordination of this effort during the early phases of drug development will help to avoid surprises later on and should facilitate the passage of the drug through the IND/NDA regulatory process.

METHOD DEVELOPMENT

The amount of time and effort put into the development of stability indicating methods for pharmaceutical dosage forms can be minimized if a logical and systematic approach is used. During the early phases of drug product development, a number of groups in a corporation are involved in the determination of the physical and chemical properties of a new drug (Figure 1). Inevitably, there will be overlap between the groups involved in this process so that free and open exchange of information between groups is a necessity in order to expedite drug development and avoid duplication of

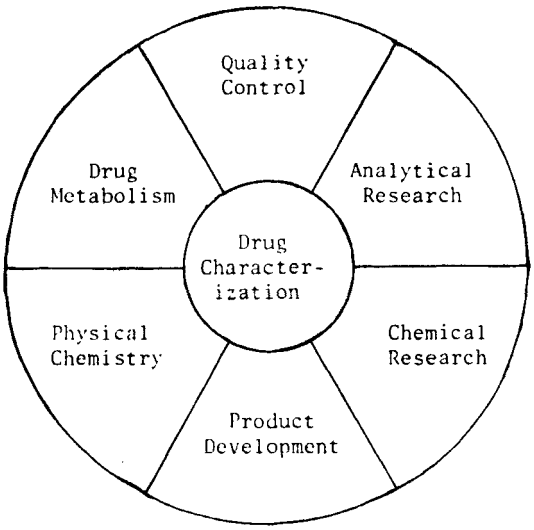


FIGURE 1: Corporate Interactions

effort. A logical and systematic approach to the development of stability indicating methods can be outlined in three basic steps: review the literature, generate background data, and develop and validate the method.

Literature Review

Before any laboratory work is started on a new drug, all of the internal information available on the drug and any existing dosage forms should be reviewed. There is almost always a trail of information available on a new drug which begins when the drug is first syntheiszed by chemical research scientists. The chemist monitors the progress of the reaction and checks the purity of the final product by some analytical procedure, typically thin layer chromatography. The physical chemistry department, while primarily interested in structure determination and confirmation, may also generate some limited analytical data, such as solubilities, spec-

trophotometric properties, and so on. Quality control or some other department generates information on the analysis of the drug per se and the drug in the vehicle used for early toxicological studies, usually including some limited stability data.

In many instances, drugs are evaluated for mutagenic potential early in development, often prior to pharmacological and toxicological screening. Since the purity of the drug may influence the outcome of the test, methods for determining purity are often developed at this stage. These may include analysis of the drug, its impurities, and degradation products as well as limited stability data on the drug in the solvent to be used for the test.

The drug metabolism department develops analytical methods to identify and measure the major metabolites of the drug as well as the parent drug in biological systems. Finally, the product development and quality control departments generate physical-chemical information as part of preformulation studies and the development of methods for the control of the drug and experimental dosage forms.

Literature sources external to the corporation should also be reviewed for information on the drug of interest as well as related compounds. If analytical methods have been published, a great deal of effort can be saved if this information is used, at least as a starting point.

Data Generation

The availability of certain basic information on the drug is invaluable in the development of stability indicating analytical methods. This includes a thorough knowledge of the physical and

TABLE 1

Physical-Chemical Characterization

Solubilities	Dissociation Constants
Partition Coefficients	Spectrophotometric Properties
Fluorescent Properties	Oxidation/reduction Potentials
Chromatographic Behavior	Stability Studies

chemical properties (Table 1) of the drug as well as identification of labile functional groups and the elucidation of degradation pathways.

Solubilities should be determined in a number of solvents covering a range of polarities which are commonly used for method development (Table 2). Solvents are added to or deleted from the list as dictated by the needs of a given drug, its dosage form, and potential methods.

Dissolution constants and partition coefficients can be used to develop efficient liquid/liquid extraction procedures, and data on fluorescent, spectrophotometric, chromatographic, and oxidation/reduction properties can be used to determine the best means of measuring and quantitating the component of interest.

Stability studies are performed on the drug "as is," in solution, and mixed with pharmaceutical ingredients as part of compatibility studies. Labile functional groups are identified and the susceptibility of the drug to hydrolytic, photolytic, oxidative, and thermal degradation is determined. Compatibility studies are performed to assess the stability of the drug when mixed with

TABLE 2

Solubility Determinations

Water	Chloroform
Aqueous Buffers	Ethyl Ether
0.1 N HCl	Cyclohexane
0.1 N NaOH	Ethyl Acetate
Ethanol	Acetonitrile

common excipients and lubricants as well as to determine the possibility of interaction of the drug with the ingredients. While routes of degradation observed with the drug per se are not always translatable to the dosage form, this information does tell you what can happen to the drug. Dosage forms can be designed to prevent or slow down degradation reactions, and, once the routes of degradation are known, they can be evaluated in order to ascertain if the same mechanisms observed in the drug are operative in the dosage form.

Method Development and Validation

Stability indicating methods for pharmaceutical dosage forms can be developed using several different approaches. Procedures can be utlized which measure the amount of drug remaining, the amount of drug lost, or single methods can be developed which separate and quantitate all known impurities and degradation products as well as the active. These last methods almost always rely on chromtography and may be difficult to handle since linearity of response is required and a computer-assisted data handling

system may be needed to quantitate accurately the wide ranges in concentrations often encountered. Alternately, the same single analytical technique may be used and quantitation achieved by varying the concentration of the sample and making more than one measurement in order to achieve a linear response and work within the constraints of the data handling system.

Separate methods may be developed, one for the quantitation of the active component, which is not necessarily specific, and the other, generally specific, for the estimation or quantitation of degradation products. Such methods may be based on the same analytical principle, e.g., chromatography, or they may utilize completely diverse techniques. The choice of the method selected depends upon the chemical nature of the drug, the complexities of the dosage form, and, often, on practical considerations relating to the availability of resources and instrumentation. If the method is to be used as a routine control test, as well as for stability studies, then practical considerations can become of paramount importance. In the majority of cases, a number of different techniques may be available for the assay of the active and degradation products, all of which yield equivalent information; the choice of the method is then made based on practical considerations.

In some instances, it may be possible to quantitate only the active or, alternately, only the degradation products, although the latter is less likely. Measurement of only the active may occur where very low potencies are encountered (in the range of mcg or

less per dose) and the limits of quantitation of available analytical techniques are not low enough for accurate measurement of degradation products. Indeed, in such instances, the elucidation and identification of degradation products and impurities can be a difficult, if not impossible, task. Also, some degradation products are formed at such low levels that isolation and identification becomes impratical.

The analytical procedures generally utilized for stability indicating methods can be divided into three general categories (Table 3).

Traditionally, the analytical methods used to monitor the stability of dosage forms have involved a generally non-specific spectrophotometric or titrimetric procedure for the assay of the active, coupled with thin layer chromatography for the estimation of impurities and degradation products and this approach is still valid. In the last five years, however, the situation has changed dramatically. Currently, the method of choice for the quantitation of the active and degradation products is increasingly becoming

TABLE 3

Stability-Indicating Methods

Electrometric	Chromatographic	Spectrometric
Titrimetric	HPLC	UV/VIS
Polarographic	GC	NMR
	TLC	Fluorescence

high performance liquid chromatography. This method has obvious advantages since it is both specific and quantitative and it lends itself well to automation. The disadvantages are that, in the absence of automation, the technique can be time-consuming, it is relatively expensive, and detection is by no means universal. Recent advances in column technology have reduced separation times to seconds, and, in the next few years, this technique may enjoy even more utility.

HPLC, however, is not the only way to go. Other chromatographic methods still find a place, particularly gas chromatography when the thermal stability of the component of interest does not pose a problem. Thin layer chromatography can be used for the rapid determination of degradation products, even when the assay of the intact drug may be performed by another chromatographic technique. Other methods may also be used, including titrimetry, polarography, fluorimetry, NMR, colorimetric analysis, etc. The choice of an appropriate method must depend on both a scientific and practical evaluation of the drug and its dosage form.

Once an analytical method is chosen, the most important aspect of the development of a stability indicating procedure is method validation [1]. Method validation should include evaluation of the following parameters:

1. Specificity--The method chosen must be shown to be capable of quantitating the component of interest. The contribution of synthesis precursors, known impurities, excipients, degradation products, or other sources of bias must be considered.

2. Linearity--The linearity of the method must be demonstrated over a range in concentrations spanning at least 50-150% of the expected work range.

3. Precision--The precision of the method and the system should be determined by the analysis of sample replicates in the first case and the repetitive measurement of a single sample in the latter.

4. Accuracy--In ascertaining the accuracy of the assay, it must be shown that all of the components of interest are recovered from the matrix with no bias. Synthetic samples of the drug in matrix spanning at least 50-150% of expected content are prepared and assayed.

5. Sensitivity--The smallest quantity of the component of interest which can be quantitated should be determined.

6. Ruggedness--The stability of analytical solutions and the variability of components of the analytical system, such as chromatographic columns, should be assessed. The test for precision should also be repeated by a second analyst using separate instrumentation, where feasible.

APPLICATIONS

The development of stability-indicating methods can best be illustrated by some practical examples.

Isotretinoin

Isotretinoin (Figure 2) is also known as 13-cis-retinoic acid and, as an analog of vitamin A, presented a challenge in the separation and identification of its degradation products since

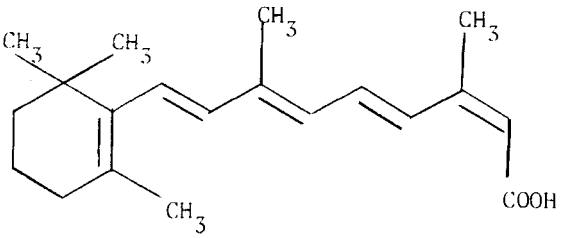


FIGURE 2: Isotretinoin

many of them are very unstable and degrade readily in the presence of oxygen and light.

A great deal is known about the chemistry of vitamin A and its analogs. The major routes of degradation are oxidation at several sites on the molecule and isomerization when exposed to light. Stability studies were conducted; they showed that isotretinoin is very stable when stored under an inert atmosphere, protected from light at room temperature. As expected with this class of compounds, in the presence of oxygen, the drug degraded significantly to a number of products within a few weeks and exposure to light resulted in the formation of primarily the all-trans isomer followed by a number of other cis isomers. Samples of the isomerization and oxidative degradation products were separated and collected using a semi-preparative HPLC system and structures were determined by NMR spectroscopy and mass spectrometry.

Four possible oxidative degradation products were identified (Figure 3). The major and first-formed oxidation product was the 5,6-epoxide (1). The 5,6-epoxide could then rearrange to the 5,8-furan (2) compound as the second most prevalent degradation

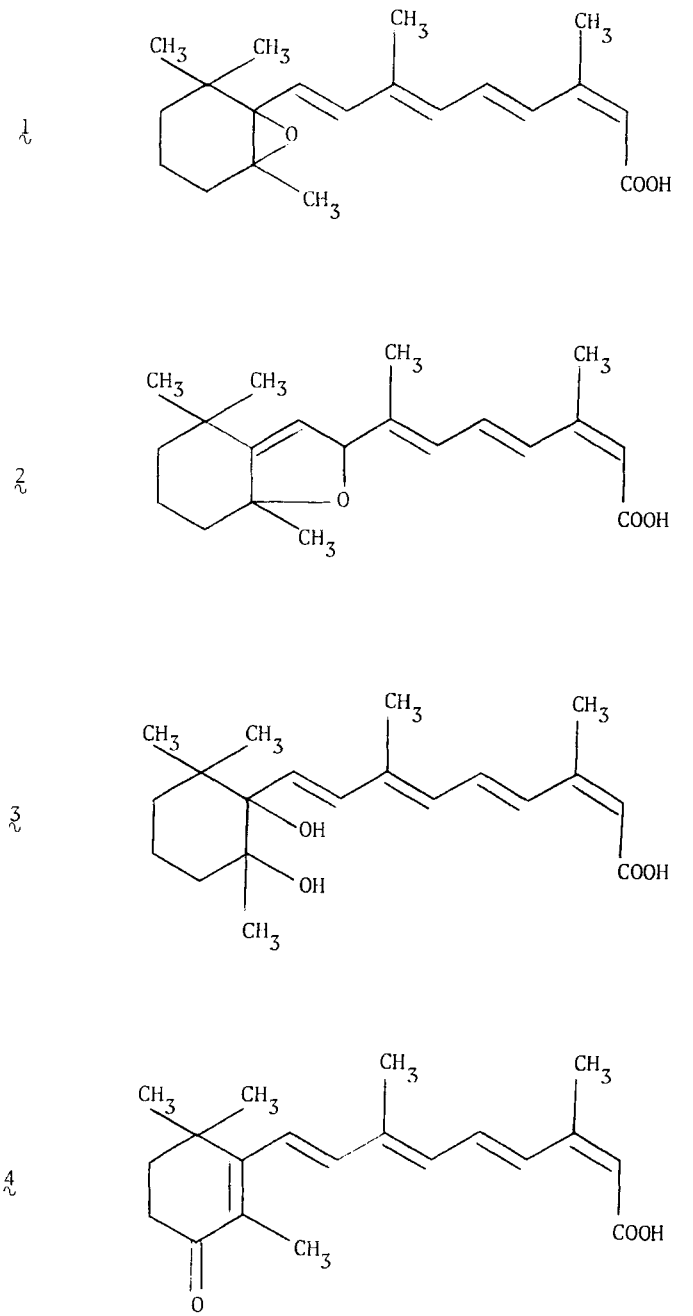


FIGURE 3: Oxidative Degradation Products

product. Hydrolytic opening of either of the epoxides (1, 2) yields the 5,6-dihydroxy compound (3). A second route of oxidation was attack at the 4 position on the ring to yield a keto degradation product (4).

Two HPLC systems were developed for the analysis of isotretinoin in capsules, one for routine control (System 1), the other for stability studies (System 2). Both methods separate all of the isomerization products from the isotretinoin; however, the first method does not separate the isomerization products from one another. In this method, the isotretinoin has a relatively short retention timne which makes the method amenable to the rapid analysis of the intact drug for content uniformity measurements. The second method may be used at all times; however, since it separates and quantitates the isomerization products, it is considerably more lengthy. Both HPLC procedures were validated with regard to the parameters mentioned above.

Specificity: The HPLC systems resolved all known degradation products and impurities from the isotretinoin, and, in the case of the second method, isomerization products and impurities were resolved from each other (Figures 4 and 5). The integrity of the isotretinoin peak was confirmed by rapidly scanning the UV-visible spectrum of the peak as it eluted from the chromatograph and comparing this spectrum to that of a reference standard. Selected peak height ratios were the same for the eluted peak from the capsule and the reference standard, within experimental error (Figure 6).

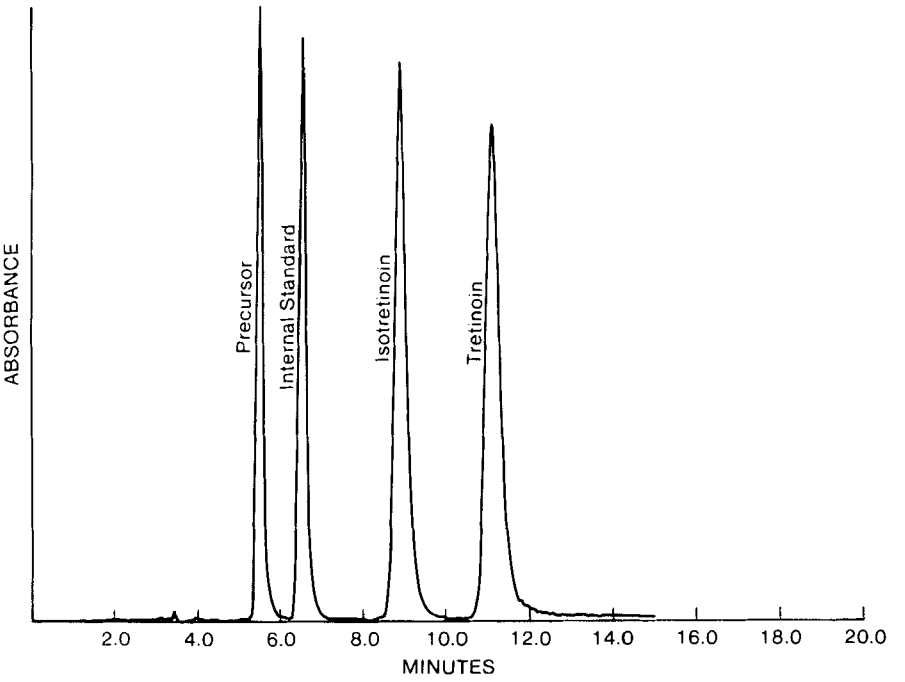


FIGURE 4: System 1.

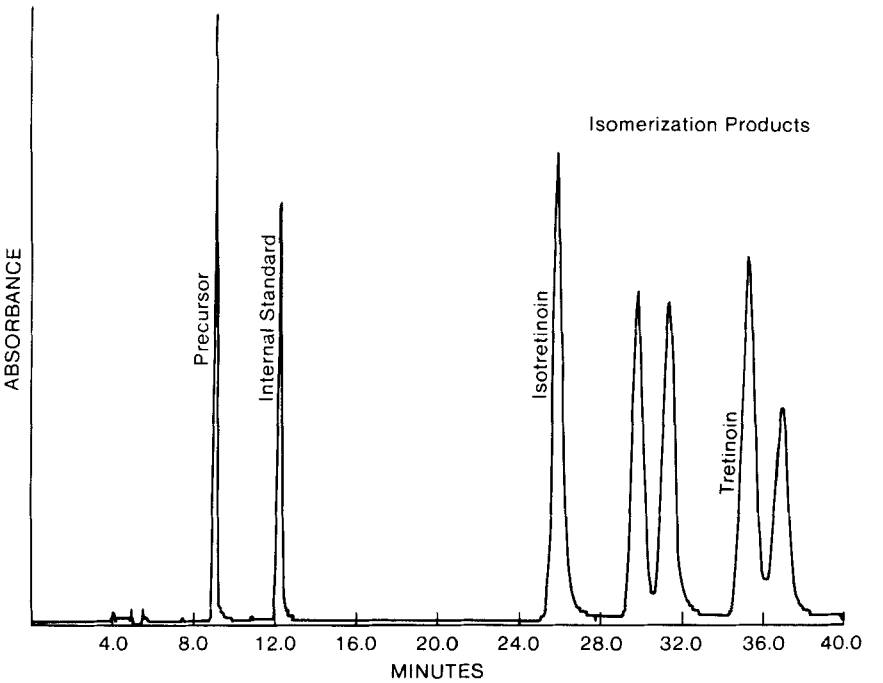


FIGURE 5: System 2.

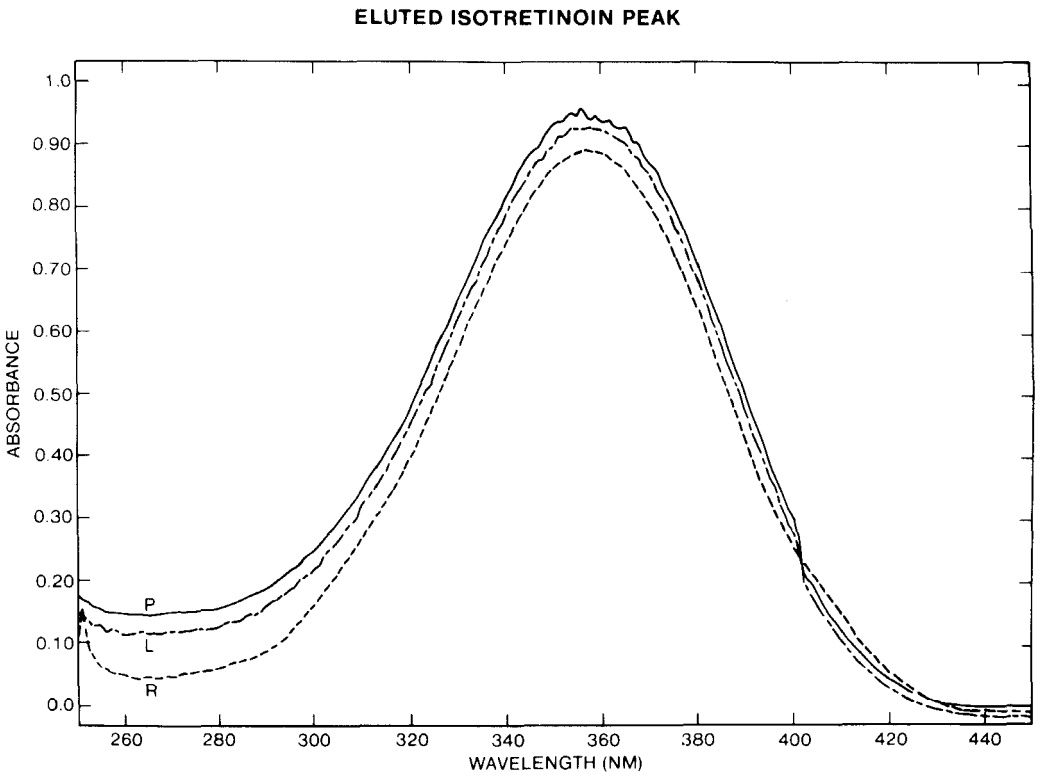


FIGURE 6: Eluted Isotretinoin peak

Linearity: The method was shown to be linear over 50-150% of the expected working range (Figure 7). The linearity was determined numerically using linear regression analysis according to Eq. (1).

Equation 1. $\log(y-b) = n \log x + \log c$ [1]

The intercept was determined to be -0.0247 and a value for n of 0.995 was obtained, which was sufficient to demonstrate the linearity of the method.

Sensitivity: The limits of detection were determined and found to be about 1 ng for the active and the isomerization products could be quantitated at the 0.1% level.

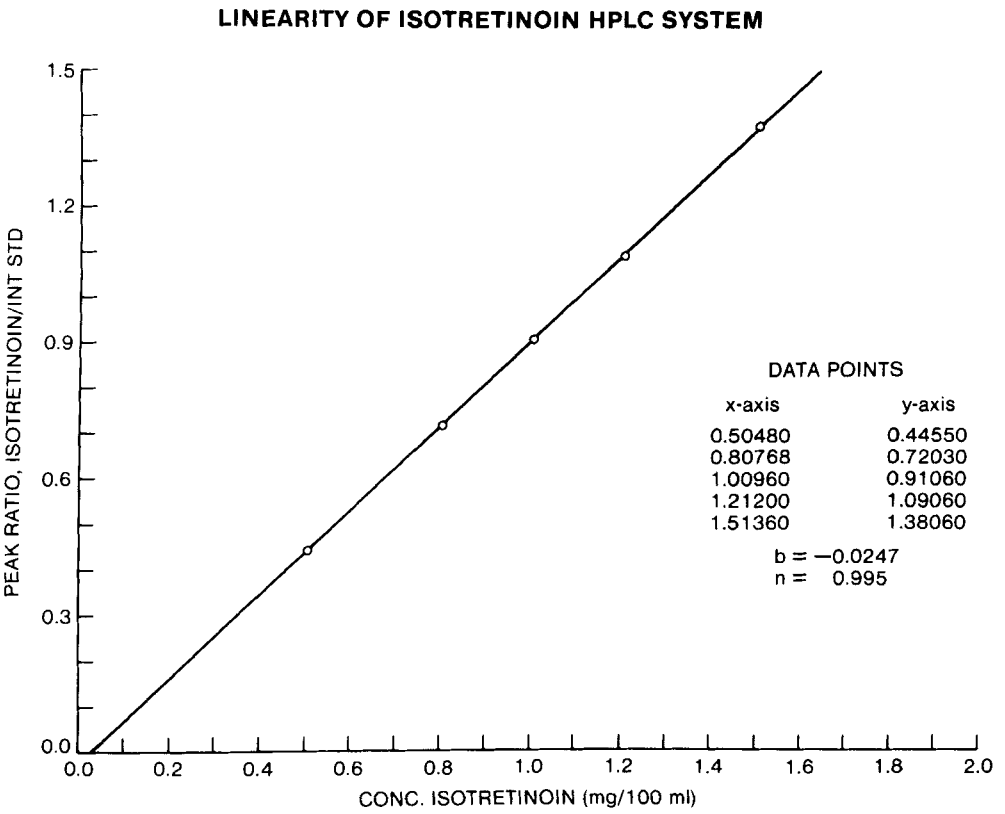


FIGURE 7: Linearity of Isotretinoin HPLC System

Precision: The entire assay procedure was carried out on 10 replicates from an analytical sample. The relative standard deviation was calculated to be 0.4% and the 90% confidence interval on an assay performed in duplicate was calculated to be 98.2-101.8%. The precision of the method was deemed acceptable for the product. A system suitability test was developed and included in the directions for testing to assure that all components of the system are functioning satisfactorily.

Accuracy: Synthetic samples of isotretinoin capsule fill were prepared, in this instance, over the range of 10-150% of label

potency. The recovery of isotretinoin from the matrix was complete with no bias.

Ruggedness: Several of the recommended columns from one manufacturer were evaluated and there was no discernible column-to-column variability in the resolution demonstrated in the assay. Columns from one other maufacturer were also found to suitable. An interlaboratory check was carried out for System 1 and there were no problems encountered in transferring the analysis to a second laboratory.

Since the oxidation products of isotretinoin are very polar, and the assay procedure was a reverse-phase HPLC method, a thin layer chromatographic method was developed to separate and quantitate these compounds should they occur in the drug. The method separates the oxidation products from isotretinoin, its precursor, and its isomerization products.

Procarbazine HCl

Procarbazine HCl is an example of a compound where an approach other than chromatography is appropriate. For compounds which are electroactive, polarography often demonstrates the necessary specificity and is a technique which should be considered.

The major route of degradation of procarbazine hydrochloride (Figure 8), both in vivo and in vitro, is oxidation of the hydrazine (—NH—NH—) center to the azo (—N=N—) moiety. Polarographic oxidation of procarbazine hydrochloride occurs at the same reactive center and thus offers a specific, stability indicating method for the intact drug. In pH 12 Britton-Robinson buffer, the

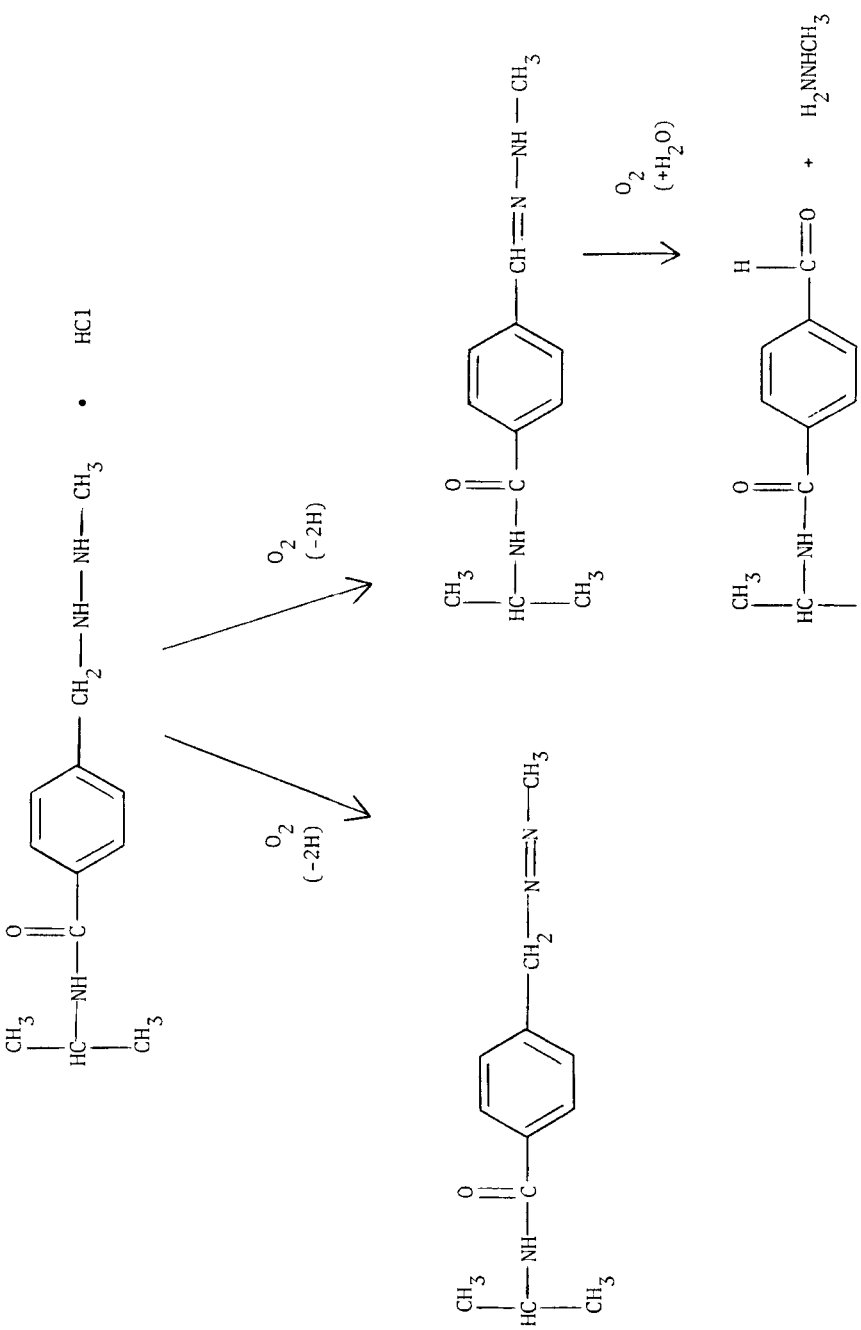


FIGURE 8: Degradation of Procarazine Hydrochloride [2]

height of the hydrazine-to-azo oxidation wave at -0.2V is proportional to the concentration of intact hydrazine group (Figure 9). The site and major product of the polarographic oxidation were confirmed by subjecting procarbazine hydrochloride to controlled potential coulometry for various time periods and polarographically analyzing the resulting solutions. As coulometric oxidation caused a decrease in the polarographic oxidation wave at -0.2V, a corresponding increase in a reduction wave at -1.4V was observed. Comparison to a known sample of azo impurity confirmed that this reduction wave was caused by the azo moiety (being formed by the coulometric oxidation of procarbazine). Secondary degradation products, a hydrazone (—CH=N—NH—) and an aldehyde, reduced polarographically at potentials more negative than the azo reduction and did not interfere with the major polarographic waves. The total number of electrons transferred in the complete coulometric oxidation of procarbazine was calculated to be two, consistent with the hydrazine-to-azo reaction. The degradation products of procarbazine HCl are independently measured using thin layer chromatography.

Midazolam

In analytical studies, NMR spectroscopy possesses the virtues of high specificity and non-destructiveness. This technique virtually ensures determination of intact compounds. Another advantage of NMR is that compounds being analyzed need not be available in pure form for use as standards. Currently, pulsed fourier transform NMR spectrometers are available where operations

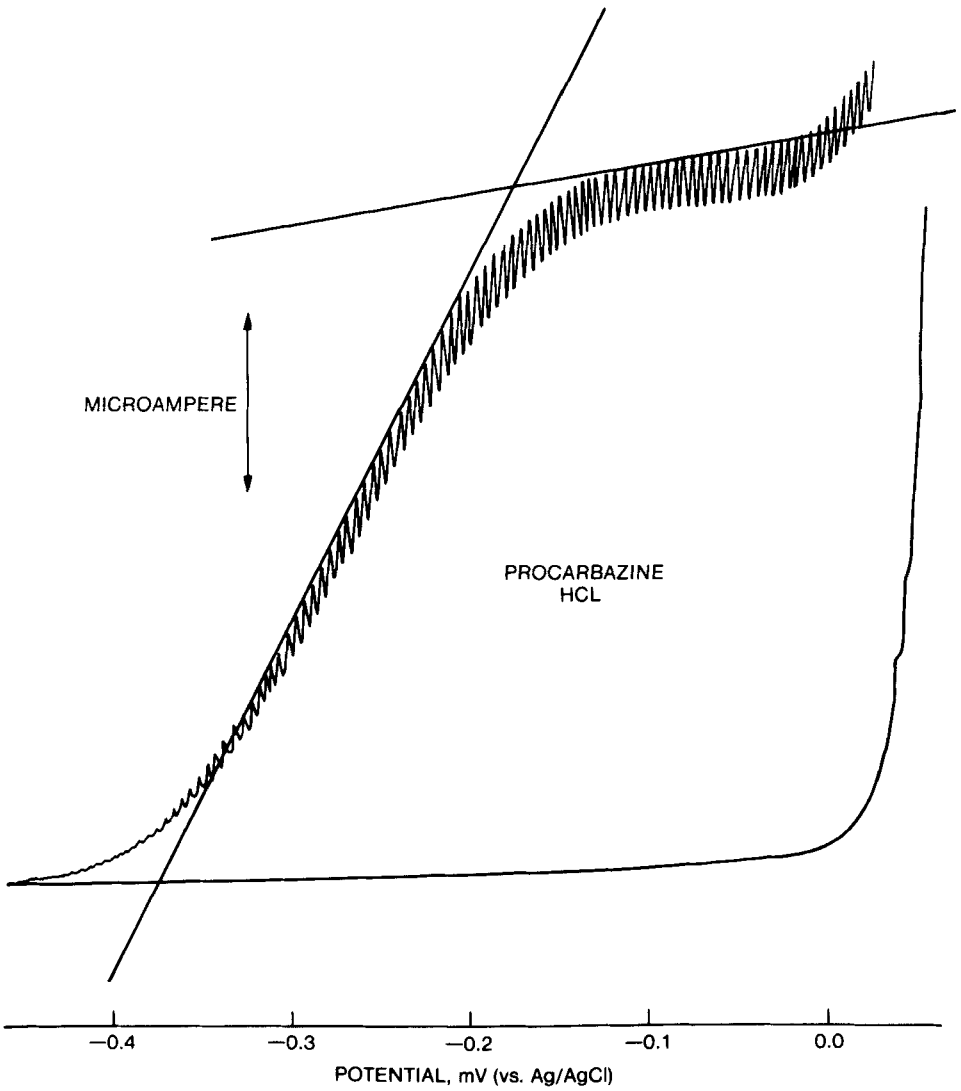


FIGURE 9: Polarogram of Procarbazine HCl

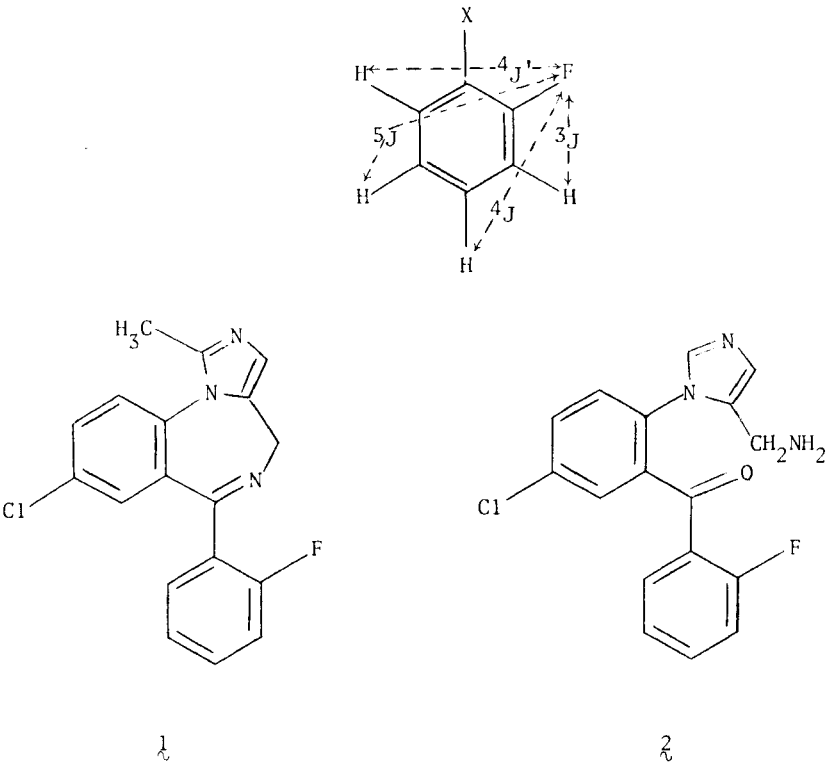


FIGURE 10: Midazolam Related Compounds

requiring high sensitivity, desirable in validating short- and long-term stability studies, can be routinely performed. NMR determinations can also play an valuable role in calibrating results obtained by less specific methods.

A typical application of the NMR technique is illustrated in the following example where fluorine-19 NMR was employed for a stability indicating determination of intact midazolam, a mono-fluoro substituted [1,4]benzodiazepine [3]. While the use of NMR is not practical for a routine control test, this study did serve to validate more conventional methods which might be employed.

Figure 10 shows the structures of the various relevant compounds in this study. Compound 1 is midazolam, the main compound of interest. Compound 2 (also referred to as the open-ring form of midazolam) exists in reversible equilibrium with 1 in protic solvents. NMR has shown that compound 2 is unstable in methanol and transforms into 1 completely in a few hours. It is important to point out that, unlike many other techniques, there are internal checks which are possible in NMR measurements. For example, the two compounds here are characterized not only by their chemical shifts (signal position) but also by their unique splitting (multiplet) patterns which serve as an additional confirmation of the identifications of the compounds derived from their chemical shifts alone in analytical studies.

An application of the NMR technique for the simultaneous stability indicating determinations of compounds 1 and 2 in a dosage form is shown in Figure 11. No sample preparation or extraction was necessary in this determination. The particular dosage form contains 5 mg/ml of compound 1 in equilibrium with 2 in an aqueous matrix (~pH 3.3). Both compounds 1 and 2 are quantitatively determined, in contrast to a previously employed colorimetric fluorescamine method where only the open-ring form, 2, is detected and the main compound of interest, 1, is totally undetected since the fluorescamine reaction takes place only with primary amines. Also, because of the pH-dependent and solvent affected equilibrium between compounds 1 and 2, HPLC and TLC methods are not suitable for intact determination of these compounds, although colorimetric

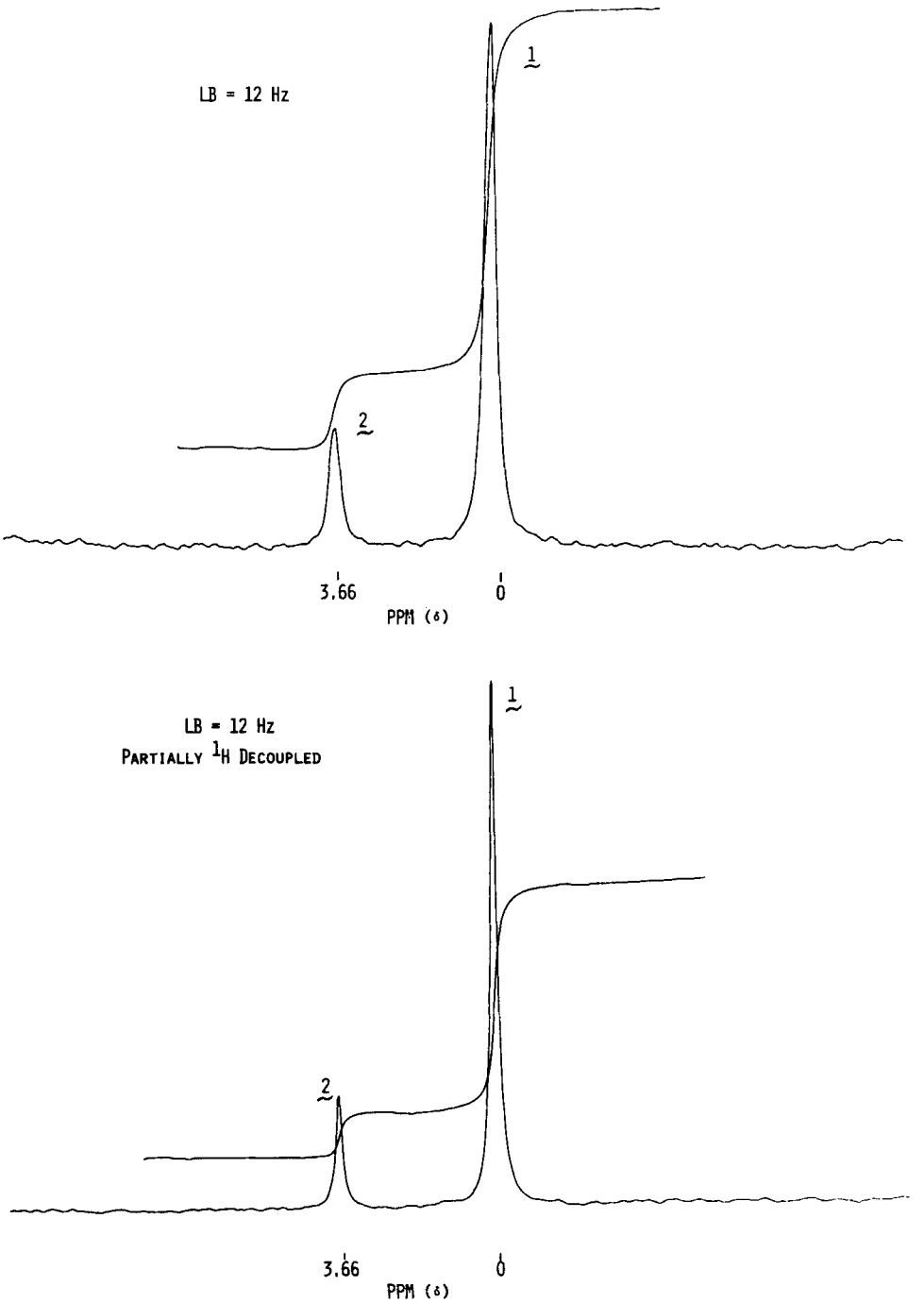
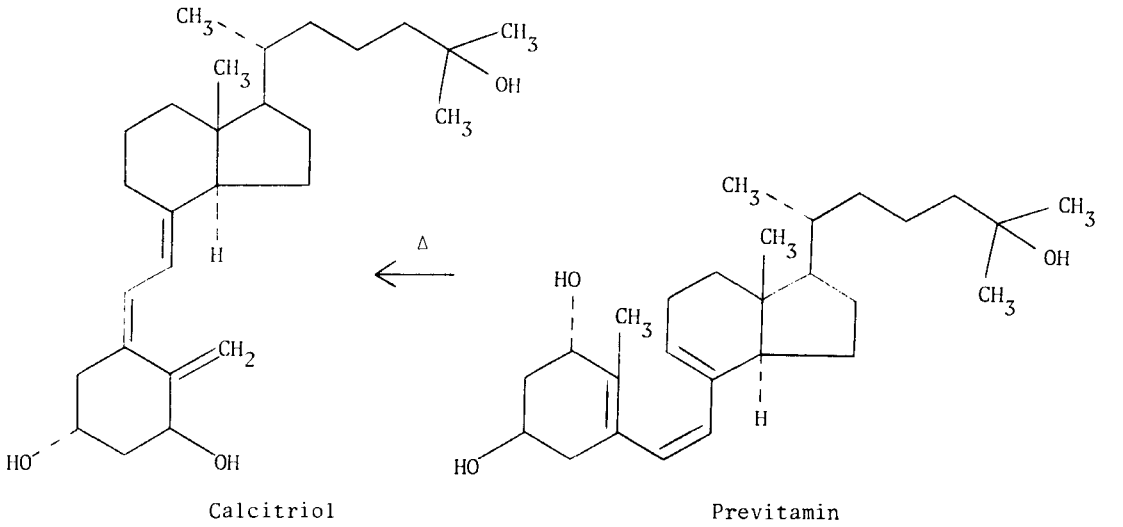


FIGURE 11: Determination of Compounds 1 and 2
The position of 1 is arbitrarily marked as "0" to show the relative chemical shift difference.

procedures can be used with ease. The NMR analysis is both specific and non-destructive, and compounds in pure form are not required for use as reference standards in this determination. The analysis is also stability indicating since expected impurites can be differentiated. Because of the unique features of the NMR technique, the NMR results can be employed in many cases to calculate calibration factors for data obtained by another technique which is non-specific for the major compound of interest (such as the colorimetric method, which is incapable of detecting compound 1 in midazolam directly). Such calibration factors can be used for subsequent routine analyses of similar samples, for example, by the colorimetric method.

Calcitriol

Calcitriol is an example of a compound where you might not quantitate all peaks in a chromatogram. High performance liquid chromatography is utilized to quantitate calcitriol, a physiologically active metabolite of vitamin D_3, in the presence of its previtamin (Figure 12) and the capsule fill matrix of its dosage form. The previtamin is not directly quantitated since it exists in equilibrium with the calcitriol and a pure reference standard is therefore not available. Direct injection of capsule fill yields a calcitriol peak which is well resolved from the matrix peaks and a previtamin peak, the area (or height) of which is proportional to concentration of intact drug (Figure 13). That the method is stability indicating was shown by heating sample solutions at several temperatures for various time periods and observing the decrease in the intact drug peak with increasing temperature and heating time.



Calcitriol Previtamin

FIGURE 12: Calcitriol and Related Compounds [4]

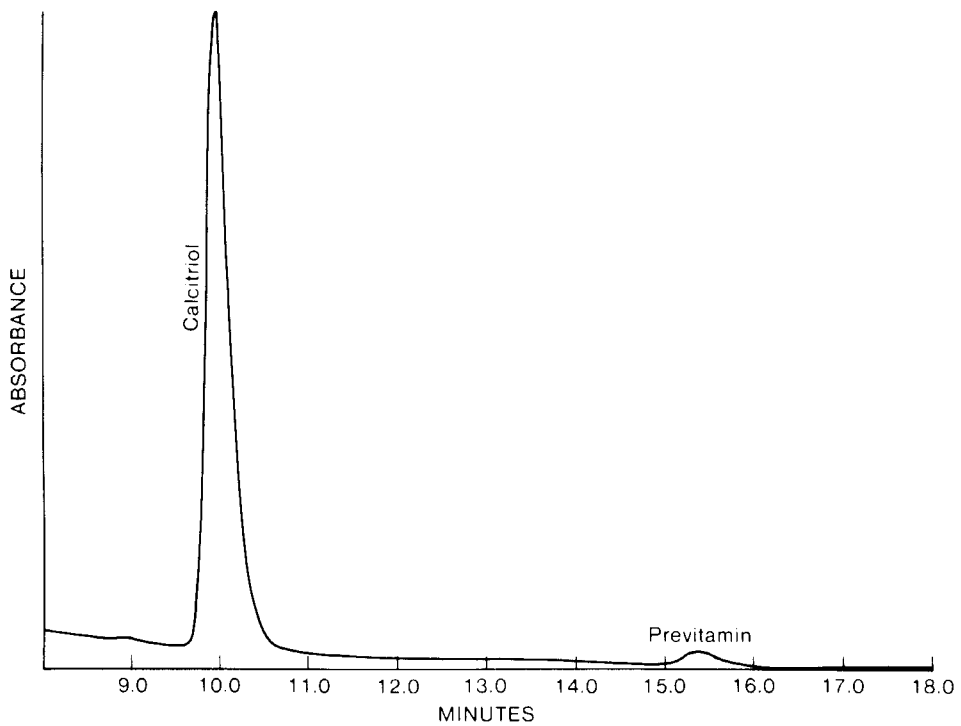


FIGURE 13: Calcitriol

The previtamin, present in small amounts in temperature-dependent equilibrium with calcitriol in solution, is not quantitated. Since previtamin converts readily to calcitriol, the thermodynamically more stable form, pure previtamin could not be isolated for use as a standard.

Diazepam

Above, it was stated that HPLC can often be quite time-consuming. Figure 14 illustrates a separation which was developed in which diazepam is separated from its metabolites in about 30 seconds. The separation, in this example, was accomplished using a microbore column and illustrates one of the directions the technique of HPLC will be going in the future.

CONCLUSION

This discussion has considered the development of methods to assess the chemical stability of pharmaceutical dosage forms. There are, of course, many other aspects to stability that could be considered, including the stability of the bulk drug and physical and organoleptic changes in a dosage form. These should be part of a separate discussion. It should be mentioned, however, that it very often happens that, during the course of product development, analytical methods evolve. As more is learned about the drug and its dosage form(s), methods can be refined and revised. No method that is developed should ever be considered final. As new technology becomes available, it is often advantageous to go back and

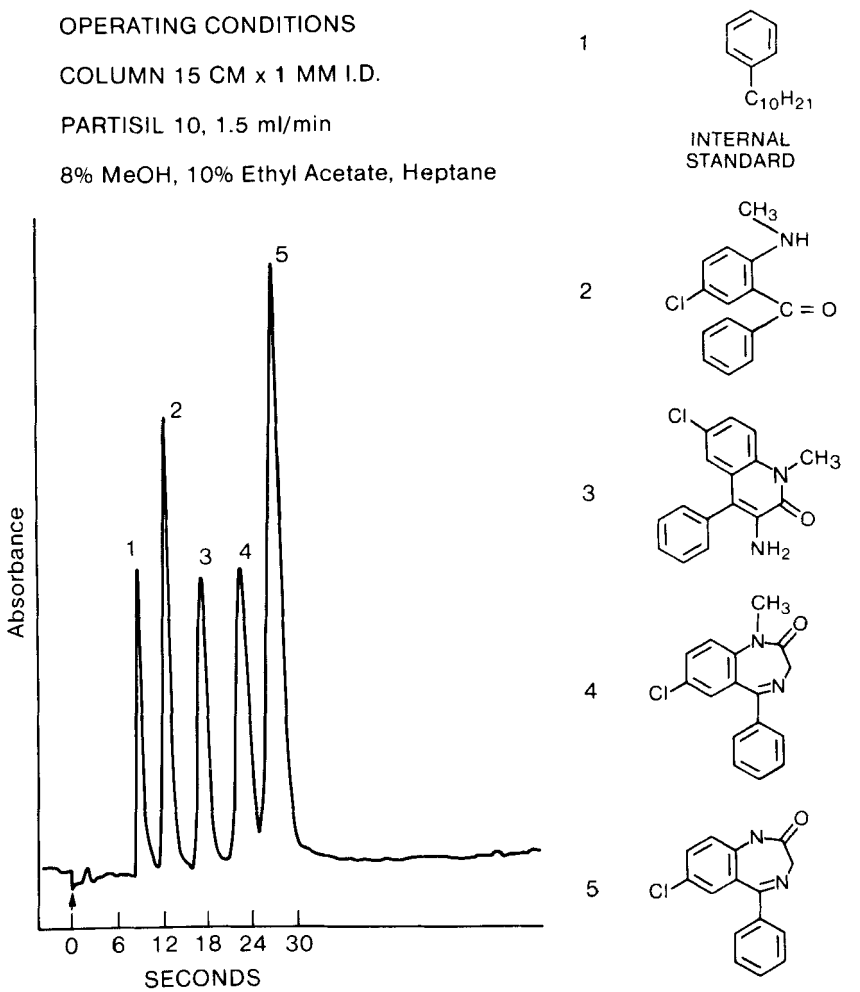


FIGURE 14: Diazepam

evaluate the methods in use and update them to the current state of the art.

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