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# The Analysis of N-Methylscopolamine Bromide by High Speed Ion Pair Partition Chromatography

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# THE ANALYSIS OF N-METHYLSCOPOLAMINE BROMIDE BY HIGH SPEED ION PAIR PARTITION CHROMATOGRAPHY

by

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# INTRODUCTION

The compound N-methylscopolamine bromide (Pamine) is a parasympatholytic drug which produces the antisecretory actions characteristic of anticholinergic drugs (1). It differs from atropine in that it has a greater selectivity in blocking vagal nerve impulses concerned with gastric secretion. It is marketed in several formulations under the trade name BIOSOL® and is designed for use in dogs, cats, and livestock to prevent and treat bacterial enteric infections and bacterial diarrheas, especially when accompanied by hypermotility and hypersecretion (2). It is formulated with the antibiotic neomycin sulfate.

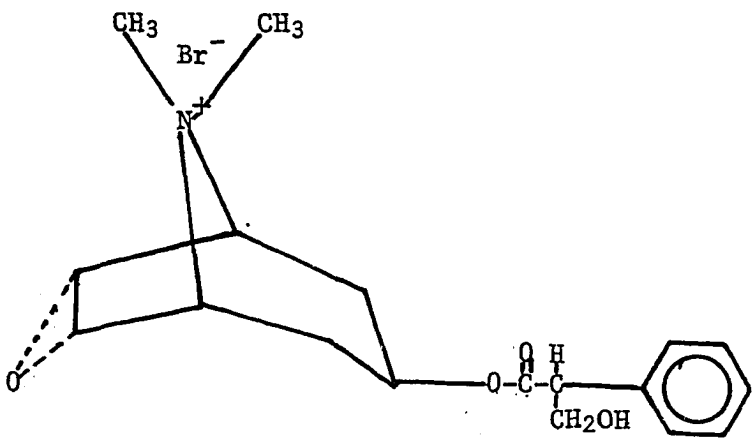
The formulation has been classified as an old drug by the FDA for many years, but a recent policy change has occurred. The FDA has reclassified all antibiotics as new drugs and since Pamine is formulated with an antibiotic, it may fall into the same category (3). As a result, a tissue residue method may be required. The target sensitivity for such a method would be 0.1 ppm.

The structure of Pamine is similar to that of atropine. It differs from atropine in that it is a quaternary ammonium bromide salt rather than a tertiary amine and it has an epoxide at the 6, 7 positions of the atropine ring. The structure of Pamine is shown in Figure 1.

The compound has a molecular weight of 398.31 and is prepared by the action of methyl bromide on the scopolamine base.

FIGURE 1

Structure of Pamine



It decomposes at 214-217°C (4). The United States Pharmacopeia standard procedure for the assay of the drug is a titrimetric method, titrating with 0.1 N perchloric acid to a crystal violet end point (5).

The method used in the determination of Pamine in formulations was originally a "dye test" where bromothymol blue formed a complex with the quaternary ammonium portion of the Pamine molecule (6). This procedure required separation of interfering components by column chromatography prior to the formation of the complex. A newer method, based on reversed phase ion pair high speed liquid chromatographic separation and detection by UV absorbance of Pamine, is presently in use. Because of the low dosage level (50 µg Pamine/ kg of body weight) the above method would not be considered satisfactory as a residue method.

The purpose of this work was to find a suitable detection system and to develop a residue method for Pamine in animal tissue

sensitive to 0.1 ppm. The major problem with this work was to find a detection method which would give the sensitivity needed for a residue method. A highly specific detection method would be desirable, but unlikely for Pamine. Thus a chromatographic procedure will probably be required to eliminate interference from natural components of the tissues.

## EXPERIMENTAL

### Methods Of Detection.

Several detection methods suitable for monitoring the effluent from a chromatographic column were tried in an attempt to determine the best method for Pamine.

Optical rotary dispersion

An aqueous solution of Pamine was prepared and analyzed on a Cary Model 60 Spectropolarimeter. The results are summarized in Table 1.

Table 1

Summary of ORD Results

| λ(nm) | Molar Rotation ([ϕ]) | Millidegrees Rotation (R) |
|---|---|---|
| 260 | -5,200 | -5 |
| 233 (min) | -15,000 | -15 |
| 212 | +26,000 | +27 |

The results of the ORD analysis indicated that there is only a relatively small rotation as compared to absorption.

Amperometric detection

Kissinger (7, 8) has successfully used his amperometric detector in a high speed liquid chromatographic (HSLC) system to detect compounds which were not easily detected by conventional means. The principle behind amperometric detection is electro-

chemical activity. The detector is a working electrode inserted in the effluent from a column. The electrode is held at a fixed potential designed to oxidize or reduce components passing by. An auxiliary electrode and a reference electrode are inserted downstream to serve as background references. A prerequisite for amperometric detection is that the compound must undergo some type of electrochemical oxidation or reduction. Tests using a Princeton Applied Research Model 174A Polargraph indicated that Pamine does not undergo either oxidation or reduction over the potential range available to the amperometric detection system (-2.0 to +1.0 V vs. SCE).

Microadsorption Detection

Another means of detecting compounds using a HSLC system is a microadsorption detector (9). The principle of operation is detection of the heat emitted due to the adsorption of a compound onto an adsorbent packed within a chamber in the detector. A second chamber within the detector is filled with a non-adsorbing packing and acts as a reference chamber. The detector used was a Varian microadsorption detector attached to a Varian Model 4000 liquid chromatograph. The detector was inserted in a plastic bag (to act as a temperature buffer) and then the bag was inserted in a Lauda K-2/R Constant Temperature Bath (Brinkman Instruments). A restrictor column was used in order to determine the sensitivity of the detector with various adsorbents to Pamine. The adsorbents were: silica gel, basic alumina, Dowex 50, neutral alumina,

Sephadex G-10, acidic alumina, Bio-Rex 70 (hydrogen form), bis-(2-ethylhexyl)hydrogen phosphate suspended on silanized Celite and dioctyl acid pyrophosphate similarly supported. The best adsorbent was Bio-Rex 70, with a 0.01 M Pamine solution giving a peak response of 18 mm. This detection system lacks the sensitivity required for a residue method.

Gas chromatograph detection

Pohlman and Cohen (10) have developed a gas chromatographic method for the determination of quaternary ammonium compounds. Their procedure involves conversion to quaternary ammonium iodides and subsequent decomposition to their respective alkyl iodides. The decomposition takes place in the injection port at a temperature of 350°C. The alkyl iodides formed in the injection port are separated on column and detected by $^{63}Ni$ electron capture. The method, as it stands, could not be used as a residue method because in a physiological media there may be several quaternary ammonium compounds which might form methyl iodides, as would Pamine, causing interference in the latter's detection. The method could be made applicable if the Pamine iodide molecule could be separated by gas chromatography intact. After separation was achieved, a post column heater could be used to generate the alkyl iodides and detection by electron capture could give the needed sensitivity.

The gas chromatograph used in this attempt at Pamine separation and detection was a Micro Tek (Tracor Industries, Baton Rouge, Louisiana) Model 220 equipped with both a flame ionization detector

(FID) and a $^{63}Ni$ electron capture (EC) detector. The column used was a 4 ft 3% OV-17 on Gas Chrom Q and the column temperature was 215°C. Attempts to prepare Pamine iodide according to the procedure of Pohlman and Cohen (10) failed. However, a 1000 ppm aqueous solution of Pamine bromide when injected on column (5 µl) gave a 40 mm peak as determined by FID. A small prepeak was observed, but no other peaks, with the exception of the solvent peak appeared. Decomposition may have been occurring, but the product observed appeared to be one major component. No peaks were observed at temperatures below 215°C. It would appear that Pamine bromide or some decomposition product can be separated on column, but the limit of detection using the FID is 250 ppm making it unacceptable as a detector in a residue method.

A post column heater was attached and the one inch space in the column surrounded by the heater was packed with copper filings to aid in the gereration of methyl bromide. The column was attached to an EC detector and the column temperature was adjusted to 215°C and the post column heater set at 400°C. Several problems were encountered in this detection system. The only solvent in which Pamine is reasonably soluble is water. It is not a good solvent for EC detection, giving a broad tailing solvent peak. The peaks observed were difficult to relate to the quantity of Pamine injected on column. The injection of 5 mg on column produced three peaks with retention times of 3.6 minutes, 4.7 minutes, and 9.0 minutes with the 9.0 minute peak being the largest. Injection of 500 µg on column also gave three peaks

with the second peak being the largest. Injection of 50 µg on column gave only two peaks with retention times of 3.5 and 4.6 minutes with the second peak again being the largest. Injection of 5 µg on column gave only one peak at a retention time of 4.6 minutes and peak height of 10 mm. The lack of linearity over the range tested was typical of EC detection and probably over a smaller range, the response would be linear. The major problem was the lack of sensitivity. A ten-fold increase in sensitivity would be required to achieve the target sensitivity of 0.1 ppm. The decomposition products were not as sensitive to EC detection as was originally expected

HSLC ion pair separation and counter ion detection

Pamine should be separable by HSLC but detection remains the problem. Its low molar absorptivity (about 189 at 257 nm, λmax) would yield low sensitivity. Since Pamine is a quaternary ammonium compound, it should easily form an ion pair with various anions. If an anion could be chosen which had high UV absorptivity, the detector sensitivity to Pamine could be enhanced considerably by such ion pair formation. Several investigators have studied the application of ion pair chromatography to pharmaceutical analysis (11-15). The basis for the separation, as they describe it, is the partitioning of an ion pair between a stationary aqueous phase, in which the pair is dissociated, and an organic mobile phase, in which the ions remain paired. The counter ion which forms the ion pair with the compound of interest is loaded on the stationary phase as

the sodium salt in a high concentration thus shifting the aqueous equilibrium to favor pairing. The ion pair, being more hydrophobic than the individual ions, partitions back into the mobile phase. The mobile phase is saturated with the counter ion salt to prevent stripping of the stationary phase. The association and dissociation of the ion pair results in the partition between the mobile and stationary phases thus giving the separation. Eksborg, et al, (11-13) used picrate and β-naphthalene sulfonate as counter ions and cellulose as the support. Karger, et al, (14, 15) used perchlorate as the counter ion and silica gel as the column support. Karger's method gave better resolution of the compounds because smaller particle sizes and higher pressures were used with silica gel. Cellulose collapses at higher pressures, thus larger particle sizes must be used so higher pressures could be avoided. Neither investigator used the counter ion to increase the sensitivity to the compound of interest as their compounds of interest were already good UV absorbers. Their use of the counter ion was merely to achieve a chromatographic separation.

Santi, Huen and Frei (16) developed an ion pair method which successfully used the picrate counter ion to enhance the detector sensitivity to scopolamine, hyoscyamine and ergotamine. Scopolamine and hyoscyamine are very similar in structure to Pamine, differing in that they are tertiary rather than quaternary ammonium compounds and hyoscyamine does not have an epoxide group.

Most investigators have used either perchlorate or picrate as

counter ions to obtain their chromatographic separations. Bromothymol blue has been used as a complexing agent with Pamine in the original formulation assay procedure (6). Methyl orange has been used as a complexing agent with atropine in a colorimetric assay (17). In this work, all of the above mentioned counter ions, with the exception of perchlorate which is UV transparent, were used in the preparation of chromatographic columns. The optimum pH for the stationary phase was determined by batch extraction of the ion pair. The column was prepared by slow addition of silica gel (Reeve Angel, 20 micron) to a 2.3 mm ID X 25 cm stainless steel one-eigth inch tubing which had been previously washed with several polar and non-polar solvents. The side of the column was tapped during the column packing procedure and the ends of the column were plugged with quartz wool. The column was loaded with the stationary phase according to the method of Karger (15). The column was washed with 100 ml of acetone and then loaded by passing an acetone: stationary phase, 1:3 solution through the column. The column was equilibrated with the mobile phase (saturated with the stationary phase) until the baseline stabilized. A pre-column was prepared in a similar manner and positioned before the injection port. All samples were injected on column as ion pairs with the appropriate counter ion. The column and pre-column were jacketed and maintained at 25°C ±0.1°C during each run. A summary of the results is shown in Table 2.

Table 2

Summary of Counter Ion Separation

| Counter Ion | pH | Column Loading (M) | Mobile Phase | Pamine on Column | Retention Time (min) | Peak Height (mm) |
|---|---|---|---|---|---|---|
| methyl orange | 5.5 | $5X10^{-6}$ | 55:45 pentanol $CH_2Cl_2$ | 200 ng | 2.9 | 20 |
| methyl orange | 5.5 | $5X10^{-5}$ | 55:45 pentanol $CH_2Cl_2$ | 320 ng | 1.7 | 51 |
| bromothymol blue | 6.0 | 0.005 | $CH_2Cl_2$ | 2 µg | no peaks observed | |
| picrate | 8.0 | 0.04 | 5% pentanol in $CH_2Cl_2$ | 50 µg | 8.4 | 13 |
| picrate | 8.0 | 0.04 | 10% pentanol in $CH_2Cl_2$ | 50 µg | 1.0 | 95 |

Methyl orange produced the best results and achieved close to the required sensitivity, but the peak was somewhat broad and attempts to sharpen the peak caused the retention time to decrease to the point where it would be useless as a separation technique. The broad peaks were indicative of poor column efficiency. A sharper peak may have been attained if the silica gel particle sizes could have been reduced to 5-10 μm. This would have required a neutral density slurry packing technique to properly pack a column; a somewhat more difficult procedure, but one which is necessary when using such small particles.

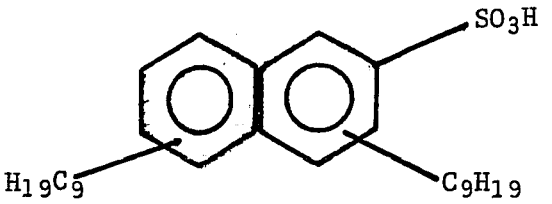
HSLC ion pair separation and Pamine detection

The final method tried was similar to that presently in use for formulation assays of Pamine. It is a reversed phase paired ion chromatographic technique developed by Wittmer, et al (18, 19). The method uses a reversed phase $C_{18}$ permanently bonded column available from Dupont Instruments (Wilmington, Delaware); a column in which octadecyl alkane is chemically bonded to silica gel particles. The mobile phase is generally 0.005 F heptane sulfonate in aqueous methanol with a small amount of acetic acid to maintain the sample in an ionic state. Heptane sulfonate forms an ion pair with the compound of interest which is partitioned into the hydrophobic stationary phase. In the stationary phase, the ion pair dissociates and is partitioned back into the aqueous mobile phase; thus affording a separation. For Pamine, the conditions established were somewhat different. The mobile phase was 0.01 F

heptane sulfonate in 9:1 (V/V) $CH_3CN$:$H_2O$ acidified with 1.25% glacial acetic acid. Pamine, injected on column under these conditions, gave a peak with a retention time of approximately 10 minutes. The sensitivity was poor, requiring one microgram on column to give a 20 mm peak at a sensitivity setting of 0.005 absorbance units full scale (AUFS). The detection limit for Pamine under these conditions thus is 100 ppm, but through sample concentration, the required sensitivity may be achieved.

## Concentration And Separation

Initially, attempts were made to extract Pamine out of an aqueous solution with two different counter ions, β-naphthalene sulfonate and 1-heptane sulfonate. Neither extracted Pamine from the aqueous phase, due to their low extraction constant for Pamine. A compound which is highly soluble in organic solutions was found readily to extract Pamine. The compound is dinonylnaphthalene sulfonic acid (DNNS). The compound is commercially available from R. T. Vanderbilt, Inc. (New York) as a 36% solution in heptane. Its exact isomeric structure is not known. The general structure is given as:




Analysis of DNNS solution by HSLC (see HSLC conditions on page 19) on a reverse phase $C_{18}$ column, gave four separate peaks. The peaks, probably representing four different isomeric structures, were isolated by silica gel column chromatography. The isolated peaks were tested for extraction efficiency for Pamine. The peak which was the most efficient at extracting Pamine was the fourth peak, as identified by HSLC. The fourth peak showed a slightly better ability to extract Pamine than the original solution, but not sufficiently better to justify the extra time in separating the different isomers. Hence, all extractions were carried out with the

commercial material without further clean-up. Extraction of Pamine from an aqueous solution into 0.005 M DNNS in methylene chloride resulted in a 95% extraction. However, a stable emulsion formed which separated only on overnight standing or by freezing and thawing. Injection of the organic extract on column (see HSLC conditions, page 19) produced a chromatogram in which the Pamine peak was on the trailing edge of one of the DNNS peaks. Higher concentration of the extractant would result in the Pamine peak being completely obscured by the DNNS peaks. To prevent this, the Pamine needed to be separated from its counter ion, DNNS. To do this, the organic ion pair solution was extracted with an aqueous solution of tetrabutylammonium acetate (TBAA). The tetrabutylammonium (TBA) ion displaced Pamine and formed an ion pair with DNNS, leaving Pamine free to be extracted into the aqueous phase. A series of extractions with varying concentrations of TBA were performed on a 1000 ppm Pamine-DNNS ion pair methylene chloride solution. The results are shown in Table 3.

The concentration of the TBAA was critical in the displacement of Pamine. To give a good extraction, TBAA had to be present in not less than equal molar amounts as DNNS, but not greater than in a two fold excess. If present in less than equal molar amounts, extraction efficiency dropped off. If present in greater than a two fold excess, the retention time of the Pamine peak decreased, giving a false peak height and causing an overlap with other peaks present in the chromatogram. Initial attempts at extraction of a

Table 3

TBA Displacement of Pamine

| TBA Concentration (M) | Peak Height (mm) | Retention Time (min) | % extracted* |
|---|---|---|---|
| 0.1 | 148 | 9.9 | 192.2 |
| 0.05 | 109 | 10.8 | 141.6 |
| 0.02 | 97 | 11.4 | 126.0 |
| 0.01 | 92 | 11.5 | 119.5 |
| 0.005 | 83 | 11.6 | 107.8 |
| 0.0025 | 14 | 12.4 | 18.2 |
| 0 | 0 | 11.6 | - |

*relative to peak height of aqueous Pamine standard

fortified liver sample failed due to the formation of a stable emulsion during the attempt at displacement of Pamine by the TBA ion. To help prevent the formation of the emulsion, a hexane/acetonitrile partition was added. The partition resulted in the extraction of the Pamine-DNNS ion pair completely by the acetonitrile phase, leaving most of the excess DNNS in the hexane phase to be discarded. The displacement of Pamine with TBA still resulted in the formation of an emulsion. A series of organic solvents were tested to find one which would not form an emulsion during the TBA displacement procedure. The solvents tested were; hexane, toluene, methylene chloride, iso-octane and ethyl acetate. Ethyl acetate was the only one which did not give

an emulsion problem. With the extraction procedure worked out, a method was developed for the analysis of Pamine in liver.

## Determination of Pamine in Liver

### Reagents

Methylene chloride, ethyl acetate, acetonitrile and acetone, distilled in glass (Burdick and Jackson, Muskegon, Michigan); dinonylnaphthalene sulfonic acid, 36% in heptane (T. J. Vanderbilt Company, Inc., New York, New York); glacial acetic acid (Mallinckrodt Chemical Company, St. Louis, Missouri); tetrabutylammonium hydroxide, 10% aqueous solution, and heptane sulfonic acid, sodium salt (Eastman Organic Chemicals, Rochester, New York).

### Procedure

A 50 g aliquot of swine liver was placed in a Waring Blendor and was fortified with an aqueous solution of Pamine. A 300 ml quantity of acetone was added to the blender. It was blended at low speed for one minute. The homogenate was filtered through a sintered glass fine porosity Büchner funnel, in which had been placed approximately 20 g of Celite 545 to act as a filter aid. The blender was rinsed three times into the funnel with small quantities of acetone. The homogenate was filtered under vacuum until the residue was dry. The filtrate was transferred to a 500 ml r.b. flask and evaporated to dryness on a high efficiency rotary evaporator (bath temperature, 40°C) (20). The residue was taken up in 50 ml of deionized water and quantitatively transferred to a 250 ml separatory funnel. The aqueous phase was extracted once with 50 ml of 0.005 M DNNS in methylene chloride. The emulsion which formed was allowed to stand overnight to facilitate phase separation. The aqueous layer was extracted twice more with 50 ml

of methylene chloride and after each extraction were allowed to separate overnight. The organic extracts were combined and evaporated to dryness. The residue was taken up in 5 ml of hexane and extracted three times with acetonitrile. The combined acetonitrile extracts were evaporated to dryness. The residue was taken up in 5 ml ethyl acetate. The ethyl acetate phase was extracted three times with 3 ml aliquots of 0.01 M tetrabutylammonium hydroxide previously adjusted to pH 3.5 with glacial acetic acid. The aqueous extracts were combined in a 15 ml screw cap tube and diluted to 10 ml with deionized water and analyzed by HSLC.

HSLC conditions

HSLC - Model 4200 (Varian Aerograph, Walnut Creek, California).

Detector - Model 440, single wavelength, UV absorbance (Waters Associates, Milford, Massachusetts).

Column - ODS, 5 micron $C_{18}$ Bondapak (Dupont Instruments, Wilmington, Delaware).

Solvent - 0.01 F heptane sulfonate in 9:1 (V/V) $CH_3CN:H_2O$ acidified with 1.25% glacial acetic acid.

Flow rate - 60 ml/hr.

Detector sensitivity - 0.005 absorbance units full scale (AUFS).

Chart speed - 0.25 in/min.

Method evaluation

The method was evaluated by three separate analysis at six different levels of Pamine. Fifty gram aliquots of liver were fortified at 0, 20, 40, 60, 80, and 100 ppm of Pamine and were processed

according to the procedure outlined on page 18. A set of six aqueous standards of 100, 200, 400, 600, 800, and 1000 ppm Pamine was analyzed along with the fortified liver samples to generate a standard curve from which the sample concentrations could be determined.

Results and discussion

The response of the detector was found to be linear, as is indicated by the linear regression analysis of the standard peak heights vs. concentration of Pamine. The results of the linear regression analysis of the three runs are shown in Table 4.

Table 4

Linear Regression Analysis of Pamine: Concentration vs. Peak Height

| | Run 1 | Run 2 | Run 3 |
|---|---|---|---|
| Correlation Coefficient | 0.9995 | 0.9980 | 0.9998 |
| Slope | 29.95 | 30.53 | 32.40 |
| Y-intercept | 0.4466 | -1.540 | -6.381 |
| Standard deviation of the estimate | 1.824 | 3.743 | 1.099 |

The correlation coefficients are equal to or higher than 0.998, indicating a linear detection response.

The results of the evaluation are summarized in Figure 2. Linear regression analysis of ppm added vs. ppm found showed the recovery to be a linear function of the concentration of Pamine with a correlation coefficient of 0.9789. The recovery was found to be 29.4% with a positive bias of 3.71 ppm. The linear analysis of ppm found is shown in Table 5.

Figure 2

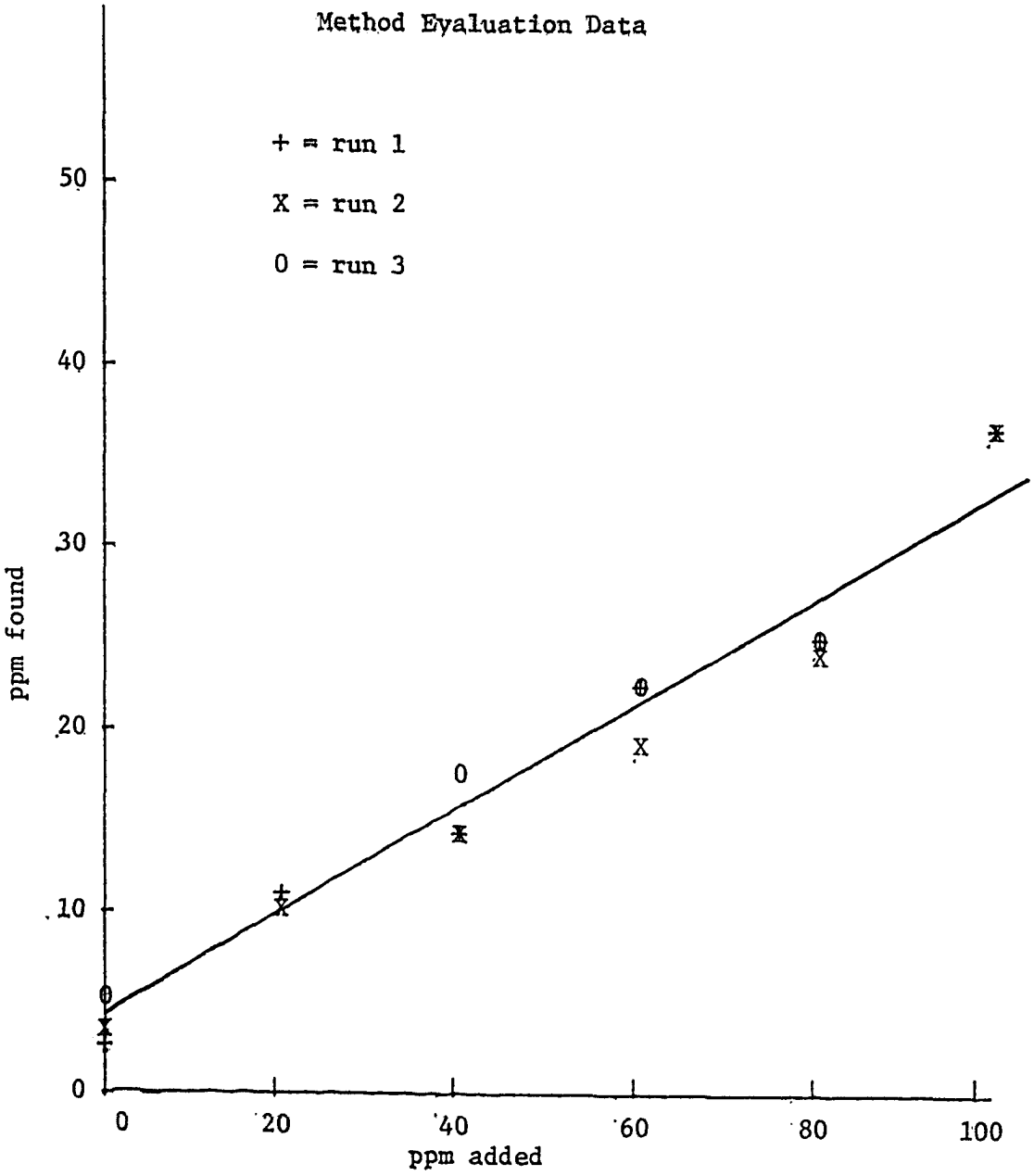


Table 5

Linear Regression Analysis; ppm Added vs. ppm Found

| | |
|---|---|
| Correlation coefficient | 0.9789 |
| Slope | 0.2941 |
| Y-intercept | 3.710 |
| Standard deviation of the estimate | ±2.110 |

## SUMMARY AND CONCLUSION

The detection methods tried, including ORD, microadsorption and amperometric detection all suffered from either lack of sensitivity or lack of activity toward Pamine. The gas chromatographic method looked promising, having a high degree of sensitivity to many compounds. The lack of sensitivity to the decomposition products of Pamine was disappointing, but could possibly be improved with a better EC detector. The problem encountered with water as a solvent could possibly be overcome by an ion pair extraction with DNNS, as done in the final ion pair method; provided the DNNS or its decomposition products did not interfere with the detection of Pamine.

Enhancement of Pamine detection by ion pairing with a highly UV absorbing counter ion would have given a highly sensitive method provided a column could have been prepared which would have given a higher separation efficiency. A more sensitive variable wavelength detector (most of the work was carried out at 0.1 AUFS) or a counter ion absorbing closer to 254 nm would have to be chosen to achieve the required sensitivity for the method even with a column capable of higher separating efficiencies. Crommen (21) developed a method for the determination of many primary, secondary, and tertiary amines with little or no UV absorbance by using β-naphthalene sulfonate as the counter ion.

The reverse phase ion pair separation and UV detection of Pamine seemed to work well in that a definite linear relationship was established between peak height and fortification level. Recovery,

however, was somewhat disappointing, being only 29.4% over the range tested. Two additional extractions with 0.005 M DNNS in methylene chloride rather than with only methylene chloride, may have helped the initial ion pair extraction. Part of the problem with the recovery was probably due to the difficulty in separating the two phases. Other organic solvents should be tried in an attempt to minimize the emulsion problem.

The reproducibility and accuracy of the analysis is acceptable for a residue method, but the detection limit is much too high, being 20 ppm. If a more sensitive variable wavelength detector were available, a slight increase in sensitivity could be achieved by increasing the detector wavelength to 257 nm, the λmax for Pamine. Also, the sample could be further concentrated, but this would also concentrate other components of the final extract. It is highly doubtful that a 200 fold increase in sensitivity could be achieved without an inordinate amount of sample clean up.

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