One sample of phenobarbital was eluted quantitatively from the adsorbent with diethyl ether containing 2% ethanol as a preservative. Prior experimental data showed that diphenylhydantoin was not eluted from the adsorbent with diethyl ether. Preparations containing sodium salts of phenobarbital and/or diphenylhydantoin would probably have to undergo acidification and extraction before column separation.

Mephobarbital and diphenylhydantoin recoveries were determined individually before synthetic mixtures were prepared. Recoveries were 98–99% for 80–115 mg. of mephobarbital and 99–101% for 40–100 mg. of diphenylhydantoin. Synthetic mixtures containing 90–150 mg. of mephobarbital and 40–100 mg. of diphenylhydantoin were then separated. Recoveries were 98 ± 0.5% for mephobarbital and 102 ± 1% for diphenylhydantoin. A small amount of mephobarbital tailing enhanced the background absorbance of diphenylhydantoin, resulting in slightly high diphenylhydantoin recoveries and low mephobarbital recoveries. This tailing could not be reduced experimentally, but its effect was minimized by subtracting the background absorbance. When recoveries were calculated with the corrected absorbances, recovery values for mephobarbital and diphenylhydantoin approached closest to 100%.

SUMMARY

An activated magnesium silicate has been used successfully to separate mephobarbital and diphenylhydantoin in a commercial tablet formulation. Application of this adsorbent to the determination of other mixtures may also yield favorable separations.

REFERENCES

(1) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 235.

(2) *Ibid.*, p. 141.

(3) F. D. Snell and C. T. Snell, "Colorimetric Methods of Analysis," Vol. IV, 3rd ed., D. Van Nostrand, Princeton, N. J., 1959, pp. 92–103.

(4) "The National Formulary," 12th ed., Mack Publishing Co., Easton, Pa., 1965, p. 142.

(5) V. S. Marino, *J. Assoc. Offic. Agr. Chemists*, **48**, 582(1965).

(6) D. Westerink, *Pharm. Weekblad*, **97**, 849(1962).

(7) I. Sunshine and S. R. Gerber, "Spectrophotometric Analysis of Drugs," Charles C. Thomas, Springfield, Ill., 1963, p. 49.

(8) C. Stainer and Y. Daube, *Duychserte-Bull. Acad. Roy Med. Belg.*, **1**, 583(1961); through *Chem. Abstr.*, **56**, 7433d(1962).

(9) "*Florisil*," Floridin Co., advertisement pamphlet, Pittsburgh, Pa.

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Determination of Niacinamide in Pharmaceutical Preparations

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Abstract □ A method for the isolation of niacinamide from multicomponent pharmaceutical preparations by partition chromatography between a diatomaceous earth-water column and chloroform followed by measurement of UV absorbance at 262 mμ is proposed. A second method using the color produced by the niacinamide in the column eluate with bromothymol blue in chloroform is also proposed to allow measurements in the visible range. The chromatographic separation is specific for niacinamide and the procedures are simple and rapid. However, alcohol modifies the partition between water and chloroform, causing the niacinamide to be eluted early. The *SD* of the UV absorbance method is 0.5% and of the bromothymol blue method is 1.7%. The precision of the proposed UV absorbance method compares favorably with that of the Pelletier and Campbell method and gives values approximately 2% higher.

Keyphrases □ Niacinamide in multicomponent products—analysis □ Partition chromatography—separation □ UV spectrophotometry—analysis □ Colorimetric analysis—spectrophotometer □ Bromothymol blue—color reagent □ Cyanogen Br-barbituric acid—color reagent

Niacinamide is an important ingredient in a variety of multicomponent pharmaceutical preparations and the great diversity of these mixtures requires a specific analytical procedure to insure accurate results. Most of the photometric methods of determination now in use are based on the Koenig reaction (1, 2) of pyridine and its derivatives with cyanogen bromide and an aromatic amine. The AOAC (10th ed.) method (3) uses sulfanilic acid and is primarily utilized for the determination of total nicotinic acid in natural products. The Pelletier and Campbell method (4) as modified by Pelletier (5) utilizes barbituric acid. Up to three times as much niacin does not interfere with this method, which has been adopted as official, first action, by the AOAC (6). The UV spectrum may also be used but is restricted to solutions containing only niacinamide unless interferences can be eliminated (7) by ion-exchange or other procedures. Polarographic (8) and GLC (9) methods have also been proposed as specific methods. All these methods suffer from disadvantages such as the use of noxious cyanogen bromide, low color stability, poor reproducibility, and interferences by other components of multivitamin preparations.

This paper reports two methods using column chromatographic separation followed by measurement of the UV absorbance of niacinamide in 0.1 *N* HCl or of the color developed by niacinamide with bromothymol blue in chloroform solution. The methods are simple, specific, and show accuracy and precision equivalent to or better than the cyanogen bromide methods.

EXPERIMENTAL[1]

Reagents—Diatomaceous earth[2] acid-washed according to the AOAC (10). Chloroform, reagent grade, washed twice with an equal volume of water and filtered through paper. Bromothymol blue (Allied Chemical), 1.20 g./100 ml. in water-washed chloroform. Niacinamide, USP reference standard, 10.0 mcg./ml. in water-washed chloroform. All other reagents were USP, ACS, or NF grade.

Determinations—The samples were run by the two proposed methods and by the method of Pelletier and Campbell as modified by Pelletier. Some of the samples were also determined using the cyanogen bromide-barbituric acid color development of the Pelletier and Campbell method after isolation of the niacinamide by the proposed chromatographic separation.

PROPOSED METHODS

Preparation of Chromatographic Column—Place a loose wad of glass wool in the bottom of the chromatographic tube. Mix thoroughly 9.0 g. of diatomaceous earth with 6.0 ml. of water and transfer in small portions (usually 6 to 8) to the column, pressing each portion evenly with a packing rod. Prepare the samples as directed below, transfer to the column, and press evenly. Dry-wash the beaker with approximately 0.5 g. of diatomaceous earth and transfer to the column. Cover with a loose pad of glass wool.

Sample Preparation—(*Syrups and Elixirs*)—Weigh, powder, and mix 20 tablets or the contents of 20 capsules. Accurately weigh a portion of the sample mixture equivalent to approximately 2.5 mg. of niacinamide into a small beaker, cover with 2.0 ml. of 5% (w/v) $NaHCO_3$, stir, add 3.0 g. of diatomaceous earth and mix thoroughly. Transfer to the column and continue as directed under column preparation.

Syrups—Transfer a 2.00-ml. aliquot to a small beaker, add 100 mg. of $NaHCO_3$ plus 3.0 g. of diatomaceous earth and mix thoroughly. Transfer to the column and continue as directed under column preparation.

Elixirs—Transfer a 2.00-ml. aliquot to a small beaker and weigh. Evaporate all the alcohol from the sample with the aid of a current of air and gentle heat. Add water to replace the alcohol until the original weight is reproduced. Add 100 mg. of $NaHCO_3$, 3.0 g. of diatomaceous earth, and mix thoroughly. Transfer to the column and continue as directed under column preparation.

Chromatographic Separation—Using a 100-ml. graduate as receiver, rinse the sample beaker with several 10-ml. portions of water-washed chloroform and add to the column. Follow with water-washed chloroform as needed to maintain a flow rate of approximately 6 ml./min. After 80 ml. of the eluate has been collected, rinse the tip of the tube with chloroform, immediately replace the receiver with a dry 250-ml. volumetric flask, and continue the elution at about 6 ml./min. Discard the eluate collected in the graduate. After approximately 245 ml. of eluate has been collected in the flask, rinse the tip of the tube with chloroform and remove the flask. Dilute to volume with chloroform.

Spectrometric Measurement—*Ultraviolet Method*—Pipet 20.00-ml. aliquots of the column eluate, the standard solution, and water-washed chloroform into separate 50-ml. glass-stoppered centrifuge tubes. Into each, pipet 20.00 ml. of 0.1 *N* HCl, shake vigorously for 30 sec., and centrifuge for 5 min. at 1,500 r.p.m. Using a medicine dropper, transfer a portion of the acid layer to a 1-cm. cell and record the spectrum of each from 320 to 240 mμ *versus* 0.1 *N* HCl on the same sheet of paper. Alternatively, the absorbance of each at 262 mμ may be measured if a recording spectrometer is not available.

The mcg./ml. of niacinamide in the eluate may be calculated from the expression

$$\frac{A_s - A_b}{A_u - A_b} = \frac{C_s}{C_u}$$

where A_s is the net absorbance of the standard at 262 mμ, A_b the net absorbance of the blank, A_u the net absorbance of the unknown, C_s the concentration of the standard in mcg./ml., and C_u the concentration of the column eluate in mcg./ml. The total weight of niacinamide in the sample is given by

$$\frac{C_u \times 250}{1{,}000} = \text{mg.}$$

This value is then converted by the usual calculation to the basis of niacinamide declared in the sample and expressed as percentage of the declared value.

Bromothymol Blue Method—Pipet 5.00 ml. of bromothymol blue solution into each of four scrupulously cleaned and dry 25-ml. volumetric flasks. Dilute one of these to volume with water-washed chloroform for use as a blank. Pipet 15.00-ml. aliquots of the column eluate into two of the flasks and 15.00 ml. of the standard solution into the fourth. Dilute all to volume with water-washed chloroform and mix well. Transfer to stoppered 1-cm. cells and rinse the outside of the cells with chloroform. Allow to stand 5 min. and measure the absorbance of the standards and samples against the blank immediately and concurrently at 430 mμ.

The mcg./ml. of niacinamide in the eluate may be calculated from the expression

$$\frac{A_s}{A_u} = \frac{C_s}{C_u}$$

where the symbols have the same significance as above. The total mg. in the original sample will be

$$\frac{C_u \times 250}{1{,}000} = \text{mg.}$$

This value is then converted by the usual calculation to the basis of niacinamide declared in the sample and expressed as percentage of the declared value.

Cyanogen Bromide-Barbituric Acid Method for Column Eluate—Evaporate 0.500-ml. aliquots of the column eluate and the standard solution just to dryness with the aid of a current of air and gentle

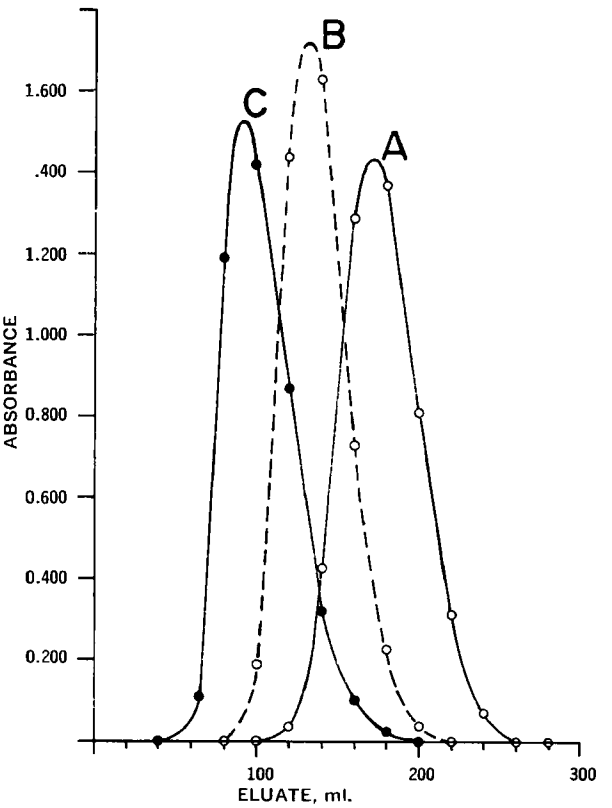

Figure 1—*Effect of alcohol on elution of niacinamide with water-washed chloroform. Key: A, no added alcohol; B, 1.5% (v/v) ethanol added; C, 3.0% (v/v) alcohol added.*

[1] The apparatus were a Cary model 15 ultraviolet spectrophotometer and a Beckman model DU spectrophotometer. Chromatographic tube, a 400-mm. length of glass tubing (i.d., 22 mm.) fused to a piece of outlet tubing.

[2] Celite 545, Johns-Manville Corp., New York, N. Y.

Table I—Assay of Niacinamide in Multivitamin Preparations By Proposed Methods and Method of Pelletier and Campbell

Multivitamin Preparations	Label Content, mg./Tablet, ml., or Capsule	Percent of Declared Value by: Proposed UV Method	Proposed Bromothymol Blue Method	Method of Pelletier and Campbell	Proposed Separation plus Koenig Reaction
Tablets	25	97.0	97.6	94.4	
		98.2	98.0		
Tablets	10	104.2		101.1	103.1
		103.2		100.5	
Tablets	20	107.1		104.5	108.4
		106.9		106.2	105.7
Tablets	100	96.5	96.8	93.8	
		96.8	95.6		
Capsules	15	83.3		82.2	80.4
		83.2			80.1
Capsules	20	106.1	107.0	103.5	
		106.6	106.9		
Capsules	25	103.7	103.8	103.2	
		105.3	107.6		
Capsules	50				
Paste contents		(73.4)[a]	(74.0)	(73.2)	
		(74.0)	(69.0)	(70.6)	
Soft gelatin shells		(29.3)	(29.3)	(28.9)	
Total (shells plus contents)		102.7	103.3	102.7	
		103.3	98.3	99.5	
Syrup	1	116.2	118.8	117.9	
		116.6	118.0	116.8	
Elixir	1	102.1	104.7	100.2	
		102.3	104.7		
Av.		102.1	104.4	100.2	
SD		0.5[b]	1.7[b]	1.4[c]	

[a] Values in parentheses not included in average and *SD*. [b] Calculated from the differences between duplicates. [c] From *Reference 4*.

heat. To each add 1.0 ml. of 0.3% (w/v) KH_2PO_4, swirl, add 0.5 ml. of 10% (w/v) cyanogen bromide and allow to stand for 25–30 min. Add 10.0 ml. of barbituric acid (2.0 g./100 ml. in 3% (w/v) KH_2PO_4) and read at 550 mμ against a blank prepared by using water in place of the cyanogen bromide. Calculate the results as for the bromothymol blue method.

METHOD DEVELOPMENT

Chromatographic Separation—The proposed chromatographic separation of niacinamide from other components depends upon the partition of niacinamide between the water of the diatomaceous earth-water column and the chloroform used as eluant. Figure 1, Curve A shows the elution of 2.00 mg. of niacinamide from the column with water-washed chloroform. The niacinamide does not appear in the eluate until approximately 100 ml. has passed through the column and the niacinamide is completely eluted in the next 200 ml. of eluate. To eliminate errors caused by normal chromatographic variation, the method has been set up to discard the first 80 ml. of eluate and to retain the next 250 ml.

In the early stages of development discrepancies in the elution characteristics of niacinamide were noted (11). Since chloroform may be stabilized with various amounts of alcohol and since the chloroform eluant used in the early studies was washed with amounts of water insufficient to remove all of the alcohol, it was decided to check the effect of alcohol on the elution. Figure 1, Curves B and C show the effect upon the elution characteristics of the addition of 1.5 and 3.0% of anhydrous alcohol, respectively, to the water-washed chloroform. The alcohol causes the niacinamide to be eluted with less volume of eluant and consequently must be removed from the chloroform before use to insure constant chromatographic conditions. In chloroform preserved with 1% alcohol and washed twice with an equal volume of water, the alcohol content is reduced to about 0.01% (12) which is satisfactory for use in the proposed methods.

Five percent sodium bicarbonate is used in the sample preparation step to convert any niacinamide acid salt to niacinamide. Hydrolysis of niacinamide at the pH of this solution is negligible (13).

Since chloroform absorbs significantly at 262 mμ, it is not a suitable solvent for the UV measurement of niacinamide. When the chloroform is removed by evaporation, losses of niacinamide occur if allowed to remain on the steam bath after the solvent has evaporated. To obviate this careful attention during evaporation, the niacinamide is extracted into 0.1 *N* HCl. The UV absorbance of niacinamide is affected greatly by the pH of the solution (13) but reaches a maximum and remains constant for pH values of 1.0 and below. Niacinamide hydrolysis is catalyzed by the hydrogen ion (13), but hydrolysis is slow in dilute acid solution. As a consequence, 0.1 *N* HCl is used as the extracting solvent in order to obtain maximum reproducible absorbance with minimum hydrolysis. Regardless, it is not advisable to allow solutions to stand for extended periods of time before measurement of the UV absorbance.

Even though niacin is usually an undesirable component in multivitamin preparations because of an unpleasant physiological reaction of some patients, it may be present. The efficiency of the proposed chromatographic separation in the elimination of niacin interference was tested by chromatographing 27- and 100-mg. quantities of niacin followed by determination by the proposed UV method. No niacin was found in the 250 ml. kept for measurement.

Bromothymol Blue Method—In an effort to provide an alternate method of measurement of the niacinamide eluted from the partition column, reaction of niacinamide with bromothymol blue was investigated. From pH 6.0 buffer, niacinamide forms a chloroform-extractable colored complex with bromothymol blue (14) but the amount of complex extracted is not sufficient for accurate quantitation. A sensitive method of measurement is provided, however, by mixing a solution of bromothymol blue in chloroform with the column eluate which is a solution of niacinamide in chloroform.

Many of the vitamins commonly occurring with niacinamide in vitamin preparations were tested qualitatively for color production with bromothymol blue. Thiamine hydrochloride, calcium pantothenate, vitamin A, and vitamin K produced a pale yellow color and riboflavin a pale orange color. Only the decomposition products from alkaline hydrolysis of thiamine hydrochloride produced an intense orange color similar to the orange color produced by niacinamide and niacin. Since the decomposition products of thiamine will interfere with the absorbance measurements in both

proposed methods, the elution characteristics were studied. A 10-mg. sample of thiamine hydrochloride was hydrolyzed at approximately 90° with 1 *N* NaOH. The base was neutralized with sulfuric acid, sodium bicarbonate and diatomaceous earth were added and mixed, and the mixture was transferred to the column and eluted in the usual manner. The first 80 ml. was collected in three 20-ml. and two 10-ml. fractions and the absorbance measured after the addition of bromothymol blue solution. The next 250 ml. was also collected and an aliquot tested with bromothymol blue solution. All interfering substances were eluted in the first 70 ml.

The optimum concentration of bromothymol blue was determined by adding bromothymol blue solutions of different concentrations to a fixed amount of a standard solution of niacinamide. It was found that the absorbance increases up to a concentration of 60 mg. of bromothymol blue/25 ml. and then remains constant up to a concentration of 110 mg./25 ml. At the concentration of 60 mg. of bromothymol blue/25 ml. used in the proposed method, the color development followed Beer's law from 0 to at least 160 mcg. of niacinamide/25 ml. A concentration of 150 mcg./25 ml. in the measured solution is equivalent to 10 mcg./ml. in the eluate or a sample size of 2.5 mg.

The bromothymol blue-niacinamide color is stable indefinitely since there was no appreciable change in absorbance of a sample measured periodically over a period of 2 weeks.

The bromothymol blue color with niacinamide is temperature-sensitive and shows a reversible absorbance decrease of approximately 3% per degree C. For this reason, the samples must be allowed to attain room temperature after transfer to the spectrometer cells and must also be read rapidly and concurrently to offset any differences between the temperature of the cell compartment and the sample.

Since traces of detergent interfere in the bromothymol blue method, the glassware used must be scrupulously cleaned with special attention paid to ground-glass surfaces. Also, since substances such as alcohol may interfere by increasing polarity of the solvent (14), care must be taken to use chloroform and glassware free of alcohol.

RESULTS AND DISCUSSION

The results of the analysis of 10 samples by the proposed methods and by the Pelletier and Campbell method are shown in Table I. Three of the samples were also determined by using the cyanogen bromide-barbituric acid method on an aliquot of the column eluate. The results by the proposed method utilizing UV absorption show a standard deviation of 0.5% based on the differences in duplicates (15) expressed as percentage of the amount declared. This value is appreciably lower than the 1.4% *SD* reported (4) for the Pelletier and Campbell method. The *SD* of 1.7% calculated for the proposed bromothymol blue method is comparable to that for the Pelletier and Campbell method.

Comparison of the results obtained by applying the specific cyanogen bromide-barbituric acid reaction to the column eluate to those obtained by the proposed UV method show that the column is efficient in separating niacinamide from interfering substances. The fact that the UV spectra of each of the ten samples was free of distortion further substantiates the efficiency of the separation.

Attention should be called to the results for the sample of soft gelatin capsules with paste contents. In this particular sample the analysis indicated that the contents contained much less niacinamide than the amount declared. Since previous work (16, 17) had shown that some types of drug ingredients may be adsorbed on or migrate into certain types of soft gelatin capsules, the empty shells were freed of any adhering material by rinsing briefly with chloroform, transferred to a 500-ml. volumetric flask, diluted to volume with 0.3% (w/v) KH_2PO_4, and allowed to stand overnight. Aliquots of this solution were run by the proposed methods and by the Pelletier and Campbell method. The results show that niacinamide equivalent to about 30% of the declared amount was contained in the capsule shells. These results indicate that the capsule shells should be checked for niacinamide content in any suspect sample.

Recovery studies utilizing 2.00-, 4.00-, and 25.00-mg. quantities of niacinamide determined by the proposed UV method showed an average recovery of 99.2% with a range of 98.7 to 99.6%. Aliquots of the 2- and 4-mg. eluates determined by the bromothymol blue method showed recoveries of 99.8 and 98.4%, respectively.

For some samples of high potency vitamins, the sample size which contains approximately 2.5 mg. is too small for satisfactory handling. In such cases, the sample size should be increased with consequent modification in the extraction step to allow adequate absorbance measurements. The recovery studies show that larger amounts of niacinamide can be chromatographed satisfactorily.

REFERENCES

(1) W. Koenig, *J. Prakt. Chem.*, **69**, 105(1904).

(2) *Ibid.*, **70**, 19(1904).

(3) "Official Methods of Analysis," 10th ed., Association of Official Agricultural Chemists, Washington, D. C., 1965, p. 763.

(4) O. Pelletier and J. A. Campbell, *J. Pharm. Sci.*, **50**, 926 (1961).

(5) O. Pelletier, *J. Assoc. Offic. Anal. Chemists*, **51**, 828(1968).

(6) "Changes in Official Methods of Analysis," *ibid.*, **51**, 506 (1968).

(7) R. Strohecker and H. M. Henning, "Vitamin Assay Tested Methods," Verlag Chemie G.m.b.H., Weinheim/Bergstr, Germany, 1965, p. 189.

(8) *Ibid.*, p. 201.

(9) A. R. Prosser and A. J. Sheppard, *J. Pharm. Sci.*, **57**, 1004 (1968).

(10) "Official Methods of Analysis," 10th ed., Association of Official Agricultural Chemists, Washington, D. C., 1965, p. 544.

(11) M. T. Jeffus, private communication, Food and Drug Administration, U. S. Dept. of HEW, 1965.

(12) C. Exley, private communication, Food and Drug Administration, U. S. Dept. of HEW, 1967.

(13) P. Finholt and T. Higuchi, *J. Pharm. Sci.*, **51**, 655(1962).

(14) V. Das Gupta and D. E. Cadwallader, *ibid.*, **57**, 112(1968).

(15) W. J. Youden, "Statistical Methods for Chemists," Wiley, New York, N. Y., 1951, p. 16.

(16) S. M. Hart, private communication, Food and Drug Administration, U. S. Dept. of HEW, 1965.

(17) B. A. Dalrymple and C. T. Kenner, *J. Pharm. Sci.*, in press.

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