# GLC Determination of Nicotinamide in Multivitamin Formulations after Conversion to Nicotinonitrile

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**Abstract** □ A method was developed for the quantitative GLC determination of nicotinamide. It is based on the conversion of nicotinamide to nicotinonitrile. This dehydration reaction is mediated with trifluoroacetic anhydride and catalyzed by base. The GLC properties of this nitrile are excellent. The reaction conditions, extraction, and stability properties of the derivative as well as the choice of internal standard are discussed.

**Keyphrases** □ Nicotinamide—GLC determination in multivitamin formulations, conversion to nicotinonitrile □ Multivitamin formulations—GLC analysis of nicotinamide, conversion to nicotinonitrile □ GLC—analysis, nicotinamide in multivitamin formulations, nicotinonitrile measured after dehydration

GLC has been widely used in the pharmaceutical analysis for the quantitative determination of various products. In the heterogeneous group of vitamins, only a few are amenable to GLC. Some approaches have been successful, particularly among the fat-soluble ones.

Nicotinamide was gas chromatographed early (1). The adsorptive properties of the compound have made the practical use of GLC methods difficult, although Prosser and Sheppard (2) and Ashby and Deavin (3) reported reproducible results. Silylation of nicotinamide was suggested also (4–6).

The present method utilizes a rapid dehydration reaction with trifluoroacetic anhydride (I) to form the nicotinonitrile and the subsequent GLC determination of that product. The GLC properties are improved considerably, permitting quantitative determination in pharmaceutical formulations with a precision of ±2.0% or less.

## EXPERIMENTAL

**Apparatus**—A gas chromatograph[1] with a flame-ionization detector was used with nitrogen (30 ml/min) as the carrier gas. The glass column (1.5 m × 0.18 cm) was filled with 20% Carbowax 20 M terephthalic acid on 100–120-mesh Gas Chrom P, acid washed and silanized. The column temperature was 155°.

**Chemicals and Reagents**—Trifluoroacetic anhydride[2] (I), pyridine, methanol, and chloroform were of analytical grade. 6-Methylnicotinamide was synthesized from 6-methylnicotinic acid[3].

The pH 1.2 buffer for extraction of tablets was prepared by mixing 2.0 g of sodium chloride, 80 ml of concentrated hydrochloric acid, and water to 1 liter.

A 0.05% solution of bromthymol blue indicator solution was prepared in water.

**Procedure**—*Standard Solution Preparation*—Weigh accurately about 25 mg of nicotinamide into a 25-ml volumetric flask. Dissolve in and dilute to volume with methanol.

Weigh accurately about 25 mg of 6-methylnicotinamide into a 25-ml volumetric flask. Dissolve in and dilute to volume with methanol. This solution is referred to as the internal standard.

*Standard Preparation*—Transfer 0.10, 0.20, and 0.30 ml of standard solution into three glass-stoppered 12-ml centrifuge tubes. Add 0.20 ml of the internal standard solution to each tube and evaporate to dryness on a boiling water bath.

Add 0.4 ml of pyridine and wait until the residue is dissolved (5–10 min). Add 0.2 ml of chloroform and 0.2 ml of trifluoroacetic anhydride. Stopper the tubes, shake gently, and let stand for 5 min in an ice bath.

Add 1 drop of bromthymol blue indicator solution and 5 *M* sodium hydroxide dropwise to a blue color. Be careful not to add an excess of the hydroxide. Cool the tubes and add 1.5 ml of chloroform. Shake for 2 min and centrifuge. Discard all of the aqueous phase, and inject about 3 μl of the chloroform layer into the gas chromatograph.

*Calibration Curve*—Construct the calibration curve by plotting the weight ratio of nicotinamide–6-methylnicotinamide on the *x*-axis against their peak height ratios on the *y*-axis.

*Sample Preparation: Liquid Formulations (1 mg/g)*—Weigh 2.0 ml of the sample in a 100-ml evaporation flask. Add 2.0 ml of the internal standard solution, and evaporate to dryness in a vacuum with a rotating evaporator on a boiling water bath.

Dissolve the residue in 1 ml of methanol. To facilitate the dissolution, warm gently and shake for 5 min. Add 9.0 ml of chloroform while shaking and then add a spoon of sodium sulfate. Filter through silanized glass wool.

Pipet 1.0 ml of the clear solution into a 12-ml glass-stoppered centrifuge tube. Proceed as described for the preparation of standards.

*Sample Preparation: Tablets (20 mg/Tablet)*—Place one tablet into a 25-ml erlenmeyer flask, and add 3.0 ml of pH 1.2 buffer. Warm and shake for 15 min in a mechanical shaker. Transfer quantitatively into a 25-ml volumetric flask, and dilute to volume with methanol.

Pipet 0.25 ml of the sample solution and 0.20 ml of the internal standard solution into a glass-stoppered 12-ml centrifuge tube. Proceed as described for the preparation of standards.

## RESULTS AND DISCUSSION

In studies of the perfluoroacylation of primary and secondary amides, Ehrsson and Mellström (7) found that primary amides were dehydrated to nitriles. This reaction has been of great value to improve the GLC properties of some primary amides studied in this laboratory. Therefore, it was of interest to apply this reaction to the quantitative determination of nicotinamide.

**Derivative Formation**—The formation of the nitrile was verified with mass spectrometry. Base catalysis was necessary according to Ehrsson and Mellström (7), who used 1.0 *M* trimethylamine in ethyl acetate. With 1 *M* triethylamine in chloroform, the dehydration of nicotinamide with I was accomplished rapidly. From a GLC point of view, pyridine was the better choice because the chromatograms were cleaner and had a much narrower solvent front.

With pyridine as the catalyst, the dehydration was very rapid, with a quantitative yield in less than 10 sec. The amount of pyridine needed for pure nicotinamide extracts was around 25 μl, giving a final pyridine concentration in the reaction mixture of 0.52 *M* (final volume ~0.8 ml).

The amount of trifluoroacetic anhydride needed for quantitative reaction was found to be 100 μl when pure nicotinamide solutions were reacted. However, these amounts of reactants were ineffective when extracts from liquid multivitamin preparations were analyzed, probably because of the poor solubility of the residue from these extracts in too small volumes of reagents. Instead, 400 μl of pyridine, 200 μl of chloroform, and 200 μl of I were used, and the dried extract was dissolved in pyridine before the anhydride was added.

[1] Varian 1400.
[2] Merck.
[3] Pfaltz and Bauer, Flushing, N.Y.

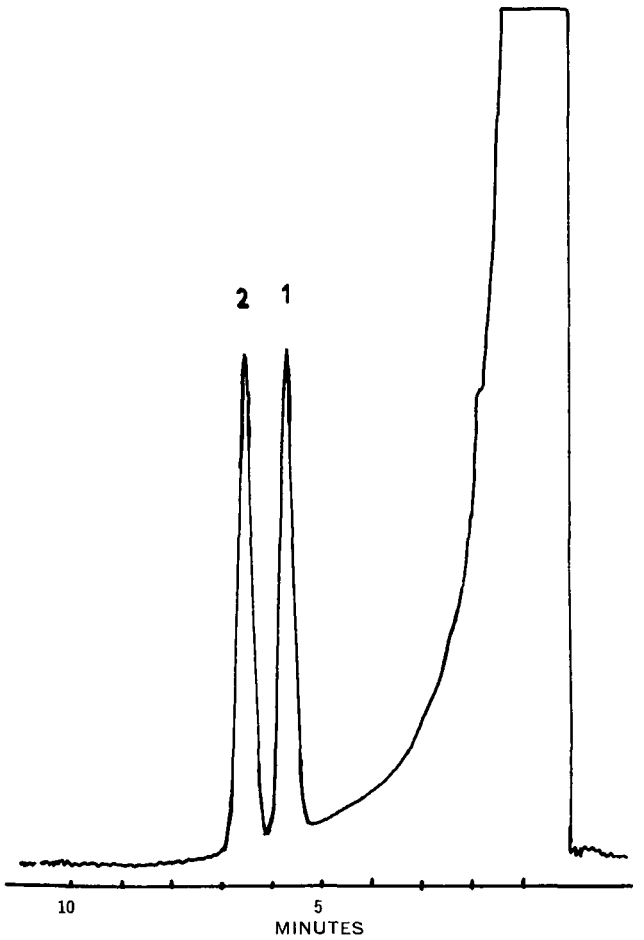


**Figure 1**—*Gas chromatogram of nicotinonitrile (1) and its 6-methyl analog (2) obtained from an extract of a liquid multivitamin preparation containing 1 mg nicotinamide/g.*

The reaction has to be done in the absence of water. Therefore, extracts or aliquots of aqueous samples were taken to dryness before the derivative formation step.

**Extraction of Nicotinonitrile**—After the dehydration reaction, it was not practical to inject the organic phase directly because the solvent front tailed immensely. Careful neutralization of the reaction mixture (indicator present), preferrably in an ice bath, and immediate separation of the phases gave an organic extract which, upon injection, gave acceptable chromatograms (Fig. 1).

Partition experiments with nicotinonitrile between chloroform and aqueous buffers showed that the extraction efficiency decreased below pH 4. Above pH 4 and with equal phase volumes, the extraction into chloroform was 94% (measured gas chromatographically against a lipophilic internal standard, decafluorobenzophenone). To obtain an extraction efficiency greater than 99%, the phase volume ratio (organic–aqueous) was adjusted to 3. To keep the volume of hydroxide needed to neutralize the trifluoroacetic acid as low as possible, 5 *M* sodium hydroxide was used. This was of particular importance in the analysis of liquid formulations. In that way the concentration of the nicotinonitrile in chloroform was kept high enough to permit the injection of reasonable volumes (≤5 μl, to keep the solvent front of reasonable size).

**Stability of Nicotinonitrile**—One source of variation was the hydrolysis of the nitriles when the reaction mixture was in contact with the alkaline aqueous phase. This was particularly apparent when a large number of samples were analyzed in a series.

Of the tested analogs to nicotinonitrile, isonicotinonitrile was most sensitive to hydrolysis. After 1 hr in contact with 5 *M* sodium hydroxide, about 25% had degraded. For nicotinamide and its 6-methyl analog, the figures were about 6 and 1%, respectively. These figures were measured against the lipophilic compound dodecane in the organic phase.

**Table I**—Relative Retention Times of Various Nitriles Derived from Pyridine Carboxylic Acids

| Substance | Nitrile in Position | Relative Retention |
|---|---|---|
| Nicotinonitrile | 3 | 1.00[a] |
| 6-Methylnicotinonitrile | 3 | 1.16 |
| 6-Chloronicotinonitrile | 3 | 2.66 |
| Isonicotinonitrile | 4 | 0.75 |
| Picolinonitrile | 1 | 1.77 |

[a] Retention time of nicotinonitrile at 155° = 6.1 min.

To minimize these hydrolysis effects, the neutralization was carefully performed in an ice bath in the presence of bromthymol blue. After centrifugation the aqueous phase was completely removed.

**GLC**—GLC properties of the nicotinonitrile were excellent in comparison with those of nicotinamide, as was recently shown by Ponomarev *et al.* (6). No signs of memory effects, *i.e.*, desorption of compounds from previous injections, were observed. The choice of Carbowax 20 M terephthalic acid as the stationary phase was chiefly dictated by the fact that this column gave the best separation of nicotinonitrile from pyridine in the solvent front (Fig. 1). After a day's use with extracts from liquid formulations, it was advantageous to exchange the first part of the column filling (2–4 cm) to inhibit clogging.

**Choice of Internal Standard**—Four analogous compounds were studied as suitable internal standards. The relative retention times of the nitriles are given in Table I. The best choice as an internal standard from a retention point of view was 6-methylnicotinamide followed by isonicotinamide. The latter would be better from an extraction point of view because the extractability was equivalent to that of nicotinamide. However, due to the greater risk for hydrolytic losses with isonicotinonitrile, the 6-methyl analog was preferred.

The dehydration of the analogous compounds was found to be as rapid as for nicotinamide.

The internal standard was added at the beginning and was present in all steps. This was of particular importance in the analysis of liquid formulations.

**Quantitative Applications**—The method has been applied on several multivitamin formulations. Tablets were extracted with an acidic buffer, and the extract was diluted with methanol to a concentration of 1 mg/ml. An aliquot of this solution was taken to dryness, and the residue was then treated with the reagents.

Liquid formulations were mixed with methanol and the internal standard solution and then taken to dryness in a vacuum. The residue was admixed with methanol and heated on a water bath for 1 min to dissolve it. Then chloroform and dried sodium sulfate were added, whereby precipitation of some constituents occurred. The chloroform layer was filtered through silanized glass wool, and then an aliquot of the extract was evaporated to dryness and processed. Sometimes the residues obtained at this point were greasy and required a couple of minutes to dissolve in pyridine.

For liquid formulations, the relative standard deviation was ±1.0%; tablets gave a somewhat higher value, ±2%. The method has been successfully used in the quality control laboratory for 1 year. This method can be applied in stability studies because the formation of the nitrile is not very probable under normal storage conditions.

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# Liquid Chromatography of Clindamycin 2-Phosphate on Triethylaminoethyl Cellulose

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**Abstract** □ The separation of clindamycin 2-phosphate from clindamycin 3-phosphate, clindamycin 4-phosphate, clindamycin B 2-phosphate, and lincomycin 2-phosphate was achieved by liquid chromatography on triethylaminoethyl cellulose using a 254-nm monitor. The compounds have low molar absorptivities at 254 nm (<17), and UV detection is made possible by the high capacity support triethylaminoethyl cellulose. Linear peak height response *versus* concentration allows rapid quantitation of clindamycin 2-phosphate.

**Keyphrases** □ Clindamycin 2-phosphate—separation from other clindamycin compounds, triethylaminoethyl cellulose column, liquid chromatography □ Triethylaminoethyl cellulose—used for liquid chromatographic separation of clindamycin 2-phosphate from other clindamycin compounds □ Liquid chromatography—separation, clindamycin 2-phosphate from other clindamycin compounds

Clindamycin 2-phosphate[1] (clindamycin phosphate) (I) is an antibacterial agent synthesized in an attempt to decrease the pain on injection associated with the compound clindamycin (1). The purpose of this work was to explore the separation of clindamycin 2-phosphate from related isomeric phosphate esters using background absorbance monitoring (254 nm), made possible by the high capacity support triethylaminoethyl cellulose.

## EXPERIMENTAL

A liquid chromatograph[2] with a 254-nm detector was used.

**Column Packing**—Triethylaminoethyl cellulose[3] was freed of fines by slurrying 1–4% suspensions in water and allowing the material to settle for 1–1.5 hr. The supernate was discarded and the slurrying–settling process was repeated six times. The purified support was isolated by filtration, washed with acetone, and air dried for 24 hr.

A funnel was attached to a 2.1-mm i.d. × 1-m stainless steel column, and 5–10 mg of triethylaminoethyl cellulose was added from an inverted container supported on a 50-mesh screen by brief mechanical vibration of the screen. The side of the column was vibrated for a few seconds, and the material within was compacted with a loosely fitting stainless steel rod (1.8-mm diameter) with an applied hand force of 0.2–0.7 kg (0.5–1.5 lb). The tamping rod was turned 120°, and three such compressions were made after each addition of support. Excessive compaction force results in columns with poor flow rates, and practice is required to produce columns with adequate flow (0.4–1.5 ml/min) using 2000 psi at room temperature.

The fully packed column was converted to the borate form by pumping about 50 ml of aqueous 0.5 *M* sodium borate (pH 8.80) through the column prior to use.

**Mobile Phase**—A solution of 0.25 *M* aqueous boric acid was prepared with addition of 10 *N* sodium hydroxide to give a pH of 8.80 ± 0.01. The solution was degassed externally for 10 min with a water aspirator while stirring vigorously.

**Samples**—Solutions of the phosphate esters were prepared at a concentration of 2–15% in water with addition of 5 *N* sodium hydroxide to give a pH of 8.80 ± 0.10. Injections of 7 μl were normally made using a 10-μl syringe[4] under continuous flow conditions.

**Peak Identification**—The identity of the collected peaks was confirmed by TLC, using silica gel plates previously sprayed with a 5% solution of boric acid, pH 8.0 (sodium hydroxide), and air dried overnight. The developing system was chloroform–methanol–water–ammonium hydroxide (40:50:6:5), and detection was achieved with a 15% ammonium sulfate spray followed by heat. The $R_f$ values were: clindamycin 2-phosphate, 0.2; clindamycin 3-phosphate, 0.4; and clindamycin 4-phosphate, 0.3.

**Phosphatase Hydrolysis (2)**—A solution of 0.05–1 mg of the

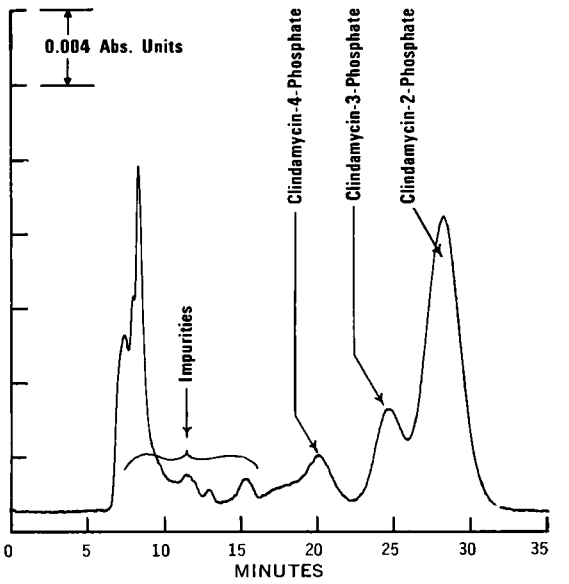


**Figure 1**—*Separation of about 0.4 mg of clindamycin 2-phosphate from a crude sample of the corresponding 3- and 4-phosphate esters, using a 2.1-mm × 1-m column of triethylaminoethyl cellulose and 0.25* M *boric acid (pH 8.80) at 60° (flow 0.23 ml/min, 410 psi).*

[1] Cleocin Phosphate, The Upjohn Co.
[2] Du Pont model 820.
[3] Cellex-T, BioRad Laboratories, Richmond, Calif.
[4] Hamilton 701N, Chaney type II adaptor.