Thin-Layer Chromatography of the Selective Rat Toxicant, Norbormide

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Norbormide has been reported to be a mixture of stereoisomers. A technique for the qualitative identification and quantitative determination of the major isomers of norbormide has been developed. Norbormide is separated into its isomers on thin-layer plates consisting of 99 per cent Silica Gel G and 1 per cent colloidal alumina, using a solvent mixture of ethyl acetate–chloroform (7:3). The isomers are extracted into 0.1 *M* hydrochloric acid–ethanol (7:1). The isomers are assayed by U. V. absorption or by the use of fluorescence. Some ultraviolet and fluorescence characteristics of the isomers are presented. Data are presented to show the accuracy and precision of the TLC–U.V. method.

NORBORMIDE,[1] 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidine)-5-norbornene-2,3-dicarboximide, has been found to be a selective rat toxicant (1, 2). As currently synthesized it consists of a mixture of stereoisomers which were separated by a variety of paper and thin-layer chromatography methods (3). These isomers were observed to vary greatly in their toxicity toward rats (4). The isomers have been classified as *cis* (S, T, W, Y) and *trans* (R, X, U, V) and further as endo (U, V, W, Y) and exo (R, S, T, X) (3). Because of this difference in biological activity, it was necessary to develop a method which would separate the isomers so that a quantitative assay of each could be carried out. Thin-layer chromatography was chosen because of the speed and sharpness of separations possible.

A thin-layer separation technique is described which separates norbormide into three isomers, designated as X, W, and Y, a mixture of two isomers Z, and a by-product in the synthesis, 7-(α-2-pyridylbenzylidine)-5-norbornene-2,3-dicarboximide (McN-1392). The mixture of the two isomers known as Z is separated further into its two isomers U and V by a second thin-layer chromatographic step. As much as 1 mg. of norbormide has been quantitatively separated into five of the eight possible racemates. Spectrophotometric and spectrofluorometric assays of the separated isomers are described.

EXPERIMENTAL

Apparatus.—The Desaga-Brinkmann complete basic equipment No. 600[2] for TLC was used for the preparation of plates. Glass plates were 2 × 8 in. A Beckman DK-2A was used for the ultraviolet absorption measurements and an Aminco-Bowman spectrophotofluorometer No. 4-8106 for fluorescence measurements.

Reagents.—Analytical reagent grade or equivalent grade reagents were used in the study. Silica Gel G was obtained from Brinkmann[2] and colloidal alumina,[3] technical grade, from E. I. Dupont de Nemours and Co., Inc.

Preparation of Plates.—A slurry of 4 Gm. of colloidal alumina and 100 ml. of water was prepared. The slurry was diluted 10 to 70 ml. with methanol. To the methanol slurry was added 40 Gm. of Silica Gel G, and the resulting slurry mixed to a uniform consistency. The glass plates were covered with a 250-μ layer of adsorbent using a fixed thickness spreader. The plates were air-dried for an hour and then at 70° for 30 min. The plates were stored in a storage rack at room temperature, 50% relative humidity, and used without any prior activation. The addition of the colloidal alumina in the silica gel provides an excellent binding with the glass surface. The use of colloidal alumina as a binder has recently been published (5).

Standard Solutions.—Solutions of the isomers of norbormide and McN-1392 were prepared by dissolving accurately weighed amounts in a 1:1 chloroform–methanol solution, and taking to a known volume. Standard solutions for the spectrophotometric analyses of the isomers were prepared by dissolving accurately weighed amounts in ethanol, and taking to volume with 0.1 *M* hydrochloric acid. The final solution was 7:1 of 0.1 *M* hydrochloric acid–ethanol.

Sample Solutions.—Samples of norbormide, norbormide isomers, and isomer mixture Z were dissolved in a 1:1 chloroform–methanol solution to obtain a concentration of 8 mg./ml. McN-1392 was dissolved to give a concentration of 2 mg./ml.

Thin-Layer Method.—Solutions of the isomers of norbormide, McN-1392, and samples of commercial norbormide were applied to the silica plate about 2 cm. from the starting edge using a Hamilton syringe pipet. For the qualitative studies from 20 to 100 mcg. of sample was applied to the plate, using a gentle stream of air to keep the spots less than 5 mm. in diameter. A maximum of 5 spots was applied across a single plate. In the quantitative studies from 800 to 1000 mcg. of norbormide was applied in a series of spots resulting in a band whose width did not exceed 5 mm.

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[1] Norbormide has been designated the American Standards Association common name.

[2] Brinkmann Instruments, Inc., Westbury, N. Y.

[3] Trademarked as Baymal.

The silica plates were developed using the ascending technique in glass jars. No paper wicks were used in the jars. Development was allowed to continue until the solvent front reached within 1 in. of the top of the plate. The plates were removed, air-dried for 5 min., and placed back into the jars for another solvent pass with the same solvent. The solvent system for the initial separation of norbormide was chloroform–ethyl acetate (7:3). For the separation of the isomer mixture Z into isomers U and V, a solution of ethyl acetate–butyl ether–acetic acid (15:5:1) was used for a total of six solvent passes. *n*-Butyl acetate can be substituted for ethyl acetate with identical results.

The separation of isomer mixture Z into isomers U and V was checked by paper chromatography (3). The separated isomers were spotted on Whatman No. 1 paper which was then sprayed with the aqueous phase of the mixture *n*-butanol–*n*-butyl-acetate–hydrochloric acid–water (75:25:6:100). The chromatogram was placed into a tank, equilibrated with the aqueous phase, and developed for 40 hr. with the organic phase of the solvent mixture.

Detection of the Isomers.—Shortwave ultraviolet light was used to locate the position of the stereoisomers on paper or silica plates. Iodine vapor or Dragendorffs' reagent can be used to detect the isomers visually on the silica plates.

RESULTS

Qualitative Analyses of the Stereoisomers.—Using the solvent system given previously for the initial separation of norbormide, commercial samples of norbormide were found to separate into 5 spots. In the order of increasing distance from the origin they were: mixture Z, isomer Y, isomer W, McN-1392, and isomer X. Further development runs did not separate mixture Z into its components. When a solvent system of chloroform–ethyl acetate (7:3) was used and 6 to 8 solvent passes, Z did separate into the isomers U and V. As the concentration of Z increased from 50 to 300 mcg. the front running spot tailed into the back spot finally resulting in a single spot. The mixture Z was separated using the butyl ether, ethyl acetate, acetic acid solvent mixture described previously. The order of separation in increasing distance from the origin was U, V. Table I lists the average distances traveled from the origin by the isomers separated from norbormide. R_f values were not calculated because of the multiple solvent pass technique. The values of the distances traveled represent the average of 40 separations of various batches of norbormide. The values given for U and V are those obtained from the separation of isomer mixture Z. Isomers R, S, and T were not found in any significant amounts in norbormide. Isomers R, S, and T could not be separated from the other isomers by the given TLC method but were separated on cellulose plates prepared with microcrystalline cellulose superfine[4] using the organic phase of the mixture *n*-butyl alcohol–hydrochloric acid–water (100:6:100) as the solvent system (3). The order of separation in increasing distance from the origin was W, V, U, and Y, R, and S, T, X.

TABLE I.—RELATIVE DISTANCES FROM THE ORIGIN OF THE ISOMERS OF NORBORMIDE AND MCN-1392 IN mm.

Sample	Isomer	Relative Distance, mm.
Norbormide	X	91 ± 4
	McN-1392	85 ± 4
	W	80 ± 4
	Y	73 ± 3
	Z	62 ± 4
Isomer mixture Z	V	78
	U	53

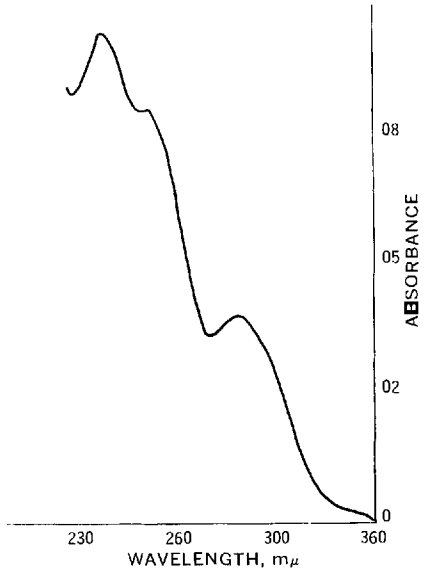

Fig. 1.—Ultraviolet absorption spectrum of norbormide in 0.1 *M* hydrochloric acid–ethanol (7:1).

Quantitative Analyses of Norbormide.—With successful resolution of norbormide into its isomers and McN-1392, the investigations were extended to studies of the resolution of mixtures for quantitative analyses. A solution containing known amounts of isomers was chromatographed, examined under ultraviolet light, and the bands outlined carefully with a sharp stylus. The adsorbent within the outlined area was scraped with a spatula onto glassine paper. The area was dry-washed with a small amount of silica gel, adding the wash to the sample. The adsorbent was transferred to a 1-oz. bottle fitted with a screw cap containing a plastic liner. An amount of 0.1 *M* hydrochloric acid–ethanol (7:1) solution was pipeted into the bottle, to obtain an approximate concentration of 0.02 mg./ml. The samples were shaken on a mechanical shaker for 1 hr., centrifuged, and the clear supernatant liquid transferred to a clean container.

Commercial precoated plates of Silica Gel G which did not contain the colloidal alumina were tried. The quantitative separations of both norbormide and norbormide isomer mixture Z were very unsatisfactory. No attempt was made to prepare TLC plates of Silica Gel G without the colloidal alumina.

Spectrophotometric Assay. The ultraviolet absorption spectrum of each sample was recorded from 360 to 230 mμ in 1-cm. cells using 0.1 *M* hydrochloric acid–ethanol (7:1) as the reference solution.

[4] Marketed as Avicel by American Viscose Division, FMC Corp., Marcus Hook, Pa.

TABLE II.—SOME ULTRAVIOLET CHARACTERISTICS FOR THE NORBORMIDE ISOMERS AND McN-1392 IN 0.1 *M* HYDROCHLORIC ACID–ETHANOL (7:1)

Sample	Max., mμ	mcg./ml./ΔA	ε	Max., mμ	ε	Absorbance Characteristic Near 260 mμ
R	295		5600	236	15,800	A peak
S	291		5200	232	13,700	Plateau
U	303	78.622	6700	239	20,800	Break
V	302	69.268	7000	239	19,300	Peak
W	300	65.329	8500	238	19,100	Break
X	295	90.073	6000	238	19,500	Break
Y	300	86.595	6400	236	13,400	Peak
Z	302	76.284	6900	238	19,500	Plateau
McN-1392	302	46.394	7500	238	17,200	Continuous

For each isomer an ultraviolet absorption curve was recorded. Approximately the same amount of Silica Gel G as in the sample was added to a volume of standard isomer solution identical to the volume of the sample. For a 1-mg. sample of norbormide 8, 4, 10, 16, and 4 ml. of the solvent was used for isomers W, X, Y, mixture Z, and McN-1392, respectively. The silica was added to the standards to match the composition of the sample solutions as closely as possible. The concentration of the isomers and McN-1392 in per cent by weight was determined by the following equation:

$$\% \text{ isomer} = \frac{(\text{mcg./}\mu\text{l./}\Delta A)_{\text{std.}} \times \Delta A_{\text{sample}} \times \text{dilution factor} \times 100}{\text{sample wt., mcg.}}$$

where ΔA, the corrected absorbance, is the absorbance of the maximum near 300 mμ minus the absorbance at 350 mμ. The ultraviolet absorption spectrum of norbormide at a concentration of 0.025 mg./ml. (1-cm. cells) in 0.1 *M* hydrochloric acid–ethanol (7:1) is given in Fig. 1. Absorption maxima are located at 300 and 238 mμ. The spectra of the individual isomers of norbormide are of the same general nature as that shown in Fig. 1. The maximum near 300 mμ was chosen to measure the concentration of norbormide since it is least affected by impurities one may encounter in a silica plate. The ultraviolet background absorbance of silica in the hydrochloric acid–ethanol solvent is linear from 360 to about 280 mμ. Since the background may vary, the absorbance near 300 mμ may be corrected for the background by subtracting the absorbance at 350 mμ.

For the purposes of identity a summary of some of the major ultraviolet absorption characteristics in 0.1 *M* hydrochloric acid–ethanol (7:1) is presented in Table II. The exo isomers, R, X, and S, have lower ε values for the higher wavelength maxima which are near 295 mμ than the endo isomers, U, V, W, and Y, at their maxima near 300 mμ. The values obtained for the factor mcg./ml./ΔA for some of the isomers are also given in Table II. They were used in the actual calculations for determining the isomer percentages given in other tables.

To test the accuracy of the assay method, three samples containing weighed amounts of the isomers W, X, Y and isomer mixture Z were assayed. The results of the assays are presented in Table III. Mixture *3* was assayed 3 times. In each case the recovery of X from mixture *3* was high. In general the recoveries of the isomers are good. Standard deviations were calculated in the usual manner.

Two samples of different lots of norbormide were assayed 6 times to test the precision of the method. The data are presented in Table IV. The 95% confidence intervals, based on the Student *t* are given for each result.

Twenty-five lots of norbormide were assayed over extended periods of time by several investigators. The mean results and 95% confidence intervals were calculated and are as follows: isomer W, 18.3% ± 4.3%; isomer X, 10.1% ± 4.3%; isomer Y, 28.7% ± 5.1%, and isomer mixture Z, 38.9% ± 4.9%. The results do not indicate significant differences in the isomer content of the different lots of norbormide.

Separation of Isomer Mixture Z.—The mixture of isomers Z was assayed by the quantitative TLC procedure already described. The composition of Z, which had been isolated and recrystallized to a constant melting point material (3), is given in Table V. Mixture Z is approximately 60% isomer V. Samples of Z separated by TLC from the other isomers in a norbormide sample were also assayed and the data given in Table V. The TLC method of separating Z from the norbormide isomers W, X, and Y has been described. However, instead of adding

TABLE III.—RECOVERY OF THE ISOMERS OF NORBORMIDE FROM STANDARD MIXTURES

Mixture *1*, mcg.	Isomers W	X	Y	Z
Taken	31	29	88	106
Recovered	37	33	86	112
Mixture *2*, mcg.				
Taken	47	44	133	159
Recovered	51	44	125	159
Mixture *3*, mcg.				
Taken	100	105	287	290
Recovered	103 ± 4	115 ± 3	274 ± 5	288 ± 16

TABLE IV.—THE COMPOSITION OF SOME NORBORMIDE SAMPLES WITH 95% CONFIDENCE INTERVAL

Sample	% W	% X	% Y	% Z
A	19.0 ± 2.8	10.1 ± 6.9	30.3 ± 2.5	34.2 ± 2.8
B	17.0 ± 4.9	12.7 ± 4.1	26.7 ± 4.4	37.3 ± 4.1

TABLE V.—COMPOSITION OF ISOMER MIXTURE Z

Sample	% U	% V
Mixture Z	36 ± 2	62 ± 3
Mixture Z[a]	37 ± 1	61 ± 2
Mixture Z[b]	40 ± 1	59 ± 1
Z[a]	14.0 ± 1.9	29.6 ± 2.4

[a] Separated by TLC from norbormide.

TABLE VI.—MOLAR FLUORESCENCE VALUES OF THE NORBORMIDE ISOMERS

Sample	Molar Fluorescence (Arbitrary Fluorescent Value Divided by the *M* Concn.)
Isomer R	3.33×10^9
Isomer S	2.49×10^9
Isomer U	5.85×10^9
Isomer V	6.51×10^9
Isomer W	2.40×10^9
Isomer X	3.47×10^9
Isomer Y	2.74×10^9
Isomer mixture Z	6.27×10^9
McN-1392	0.82×10^9

the 7:1 mixture of 0.1 *M* hydrochloric acid–ethanol, 25 ml. of methanol was added and the sample shaken for 1 hr. on a mechanical shaker. The sample was centrifuged and exactly 20 ml. of the clear solution was evaporated to dryness without the aid of heat. The residue was quantitatively taken up in methanol and streaked on the silica plates. The silica plate was developed as previously described.

Paper chromatography was used to determine the sharpness of separation of U and V. If the silica plates were not kept at 25° and 50% relative humidity there was from 5 to 10% tailing of the front running band V into the slower band U. The data given for Z separated from the other isomers by TLC represents separations from 5 different lots of norbormide. The standard deviations are also given in the table. Isomer mixture Z, obtained by recrystallization techniques, was found to contain U and V in the ratio of about 4:6 while, mixture Z separated from samples of norbormide by TLC contained them in the ratio of about 1:2.

Fluorescence Assay.—The norbormide isomers were also assayed by measurement of their fluorescence in acid. All the isomers have an activation maximum at 320 ± 5 mμ and a fluorescence maximum at 460 ±5 mμ in a 1:7 ethanol–0.1 *M* hydrochloric acid solvent. When arbitrary fluorescence units were plotted against concentration, a straight line relationship was found for each isomer in the concentration range of 1 to 10 mcg./ml. The method of assay is the same used for the spectrophotometric assay except that the sample dilution is increased. Standards containing approximately the same amount of silica as the samples are taken through the sample extraction procedure with 1:7 ethanol–0.1 *M* hydrochloric acid. Because of the care needed to avoid traces of impurities anywhere in the fluorometric assay, the spectrophotometric assay is preferred. Good agreement has been observed between the fluorescence and spectrophotometric assays for samples assayed by both methods. The molar fluoresence, arbitrary fluorescence units divided by the molar concentration, is given in Table VI for the major isomers of norbormide.

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Ion-Pair Extraction of Pharmaceutical Amines II

Extraction Profile of Chlorpheniramine

By TAKERU HIGUCHI and K. KATO

The extractability of chlorpheniramine in its ion-pair form has been investigated under several conditions to determine the suitability of the process for separation and isolation of the drug in analytical samples. Chlorpheniramine, chosen as an example of a drug having two basic centers per molecule, exists in aqueous solution as a mixture of uncharged, singly charged, and doubly charged species. Data are presented to show that the drug can be extracted as the chloride, bromide, maleate, trichloroacetate, picrate, etc. The extraction-pH profiles of both the picrate and the bromide correspond closely with the theoretical relationship. As with the monoacidic amines, extraction into the organic phase requires the presence of proton donating species. Experimental data suggest, for example, that the extracted species is coordinated with 5 molecules of chloroform.

THE EFFECTS of various anions and the dependence on solvating agents present in the organic phase on the formation of ion pairs extractable into lipoidal solvents were previously presented (1), with dextromethorphan as an example of a monoprotic amine. The current work deals with the behavior of a diprotic organic base, chlorpheniramine.

It has been found that, in addition to showing a marked dependency on the masking or complex-

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