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GLC Determination of Tilidine, Nortilidine, and Bisnortilidine in Biological Fluids with a Nitrogen-Sensitive Detector

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Abstract □ A sensitive and specific GLC method is described to determine therapeutic levels of tilidine and its two main metabolites, nortilidine and bisnortilidine, in plasma and urine. The method involves the extraction of the compounds and an internal standard with cyclohexane from alkalinized samples, followed by back-extraction into 1 *N* HCl. The hydrochloric acid solution is evaporated to dryness. After liberation of the free bases with ammonia, the residue is subjected to GLC analysis with a nitrogen–phosphorus detector and a 1.8-m (6-ft) glass column packed with 1% CRS 101 and 1.5% LAC-4-R-886 on Gas Chrom Q. Sensitivity in plasma and urine is ~1 ng/ml for a 5-ml sample.

Keyphrases □ Tilidine and metabolites—GLC analyses in biological fluids □ GLC—analyses, tilidine and metabolites in biological fluids □ Analgesics—tilidine and metabolites, GLC analyses in biological fluids

Tilidine hydrochloride[1] [ethyl DL-*trans*-2-(dimethylamino)-1-phenyl-3-cyclohexene-1-carboxylate hydrochloride] is a potent analgesic (1). Tilidine is metabolized rapidly to form the corresponding *N*-desmethyl- and *N*-bis(desmethyl) derivatives (2). A specific and sensitive analytical method was needed for the quantitative determination of tilidine and its two main metabolites, nortilidine and bisnortilidine, in biological fluids to study their kinetics in the body.

A GLC method (3) was used in previous pharmacokinetic studies, but the GLC column exhibited adsorption of bisnortilidine after a short time. A modified GLC column, well suited for the analytical control of the substances, was described recently (4).

This paper reports a new, sensitive, and selective GLC assay for tilidine, nortilidine, and bisnortilidine in plasma and urine. A thermoionic detector and a column packed with 1% CRS and 1.5% LAC-4-R-886 on Gas Chrom Q are used. The advantages of this method in comparison to the previous method are improved column stability, increased

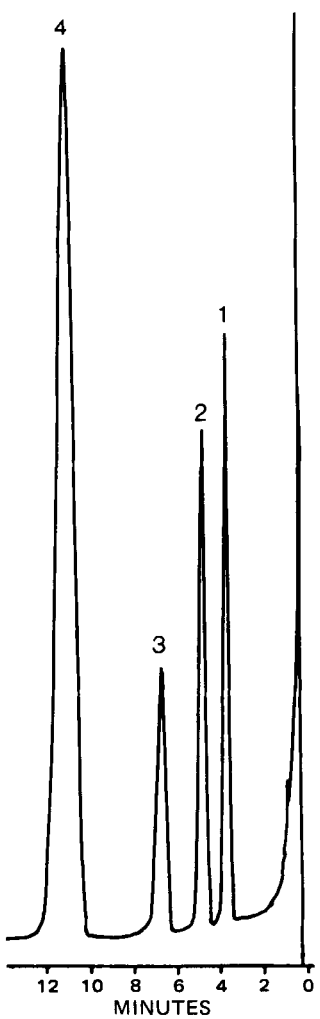

Figure 1—*Chromatogram of standards directly injected onto the columns. Key: 1, 20 ng of tilidine; 2, 20 ng of nortilidine; 3, 20 ng of bisnortilidine; and 4, 150 ng of internal standard. Attenuation was 16 × 1.*

[1] Valoron, Gödecke AG, Freiberg, West Germany (IND approved in the United States).

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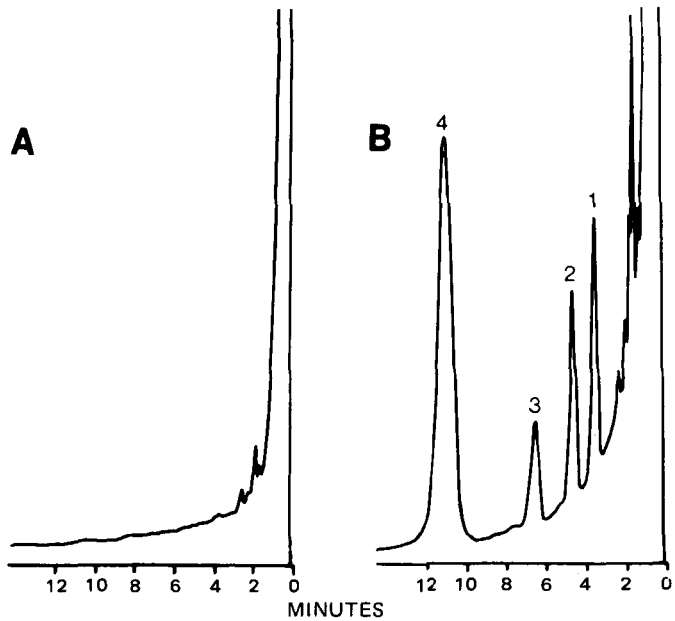

Figure 2—*Chromatograms of a blank plasma extract (A) and an extract from 10 ml of plasma spiked with 50 ng of tilidine, nortilidine, and bisnortilidine and 500 ng of internal standard (B). Key: 1, tilidine; 2, nortilidine; 3, bisnortilidine; and 4, internal standard. Attenuation was 16 × 1.*

sensitivity, and simplified cleanup. The utility of the method is shown after administration of 50 mg iv of tilidine hydrochloride to humans.

Table I—Peak Area Ratios (Tilidine, Nortilidine, and Bisnortilidine *versus* Internal Standard) after Three Calibration Runs with Spiked Human Plasma

Amount, ng	Peak Area Ratio						
	Calibration						
	1	2	3	Mean	*SD*	*CV*, %	*SEM*
			Tilidine				
25	0.0850	0.0930	0.0880	0.0887	0.0040	4.56	0.0023
50	0.1800	0.1900	0.1700	0.1800	0.0100	5.56	0.0058
100	0.4100	0.4400	0.3600	0.4033	0.0404	10.02	0.0233
250	1.1000	1.2050	1.0500	1.1183	0.0791	7.07	0.0457
500	2.0500	2.1800	2.0500	2.0933	0.0750	3.59	0.0433
1000	3.7500	4.0800	3.9700	3.9333	0.1680	4.27	0.0970
			Nortilidine				
25	0.0920	0.1000	0.0890	0.0937	0.0057	6.07	0.0033
50	0.1950	0.2100	0.1800	0.1950	0.0150	7.69	0.0087
100	0.4100	0.4300	0.4000	0.4133	0.0153	3.70	0.0088
250	1.1500	1.0800	1.0500	1.0933	0.0513	4.69	0.0296
500	2.2100	2.3000	2.1600	2.2233	0.0709	3.19	0.0410
1000	4.0700	4.2800	4.1200	4.1567	0.1097	2.64	0.0633
			Bisnortilidine				
25	0.0680	0.0820	0.0650	0.0717	0.0091	12.66	0.0052
50	0.1400	0.1600	0.1400	0.1467	0.0115	7.87	0.0067
100	0.3000	0.2800	0.3200	0.3000	0.0200	6.67	0.1155
250	0.7200	0.7100	0.7700	0.7333	0.0321	4.38	0.0186
500	1.4100	1.4800	1.5200	1.4700	0.0557	3.79	0.0321
1000	2.7800	2.9500	2.9800	2.9033	0.1079	3.71	0.0623

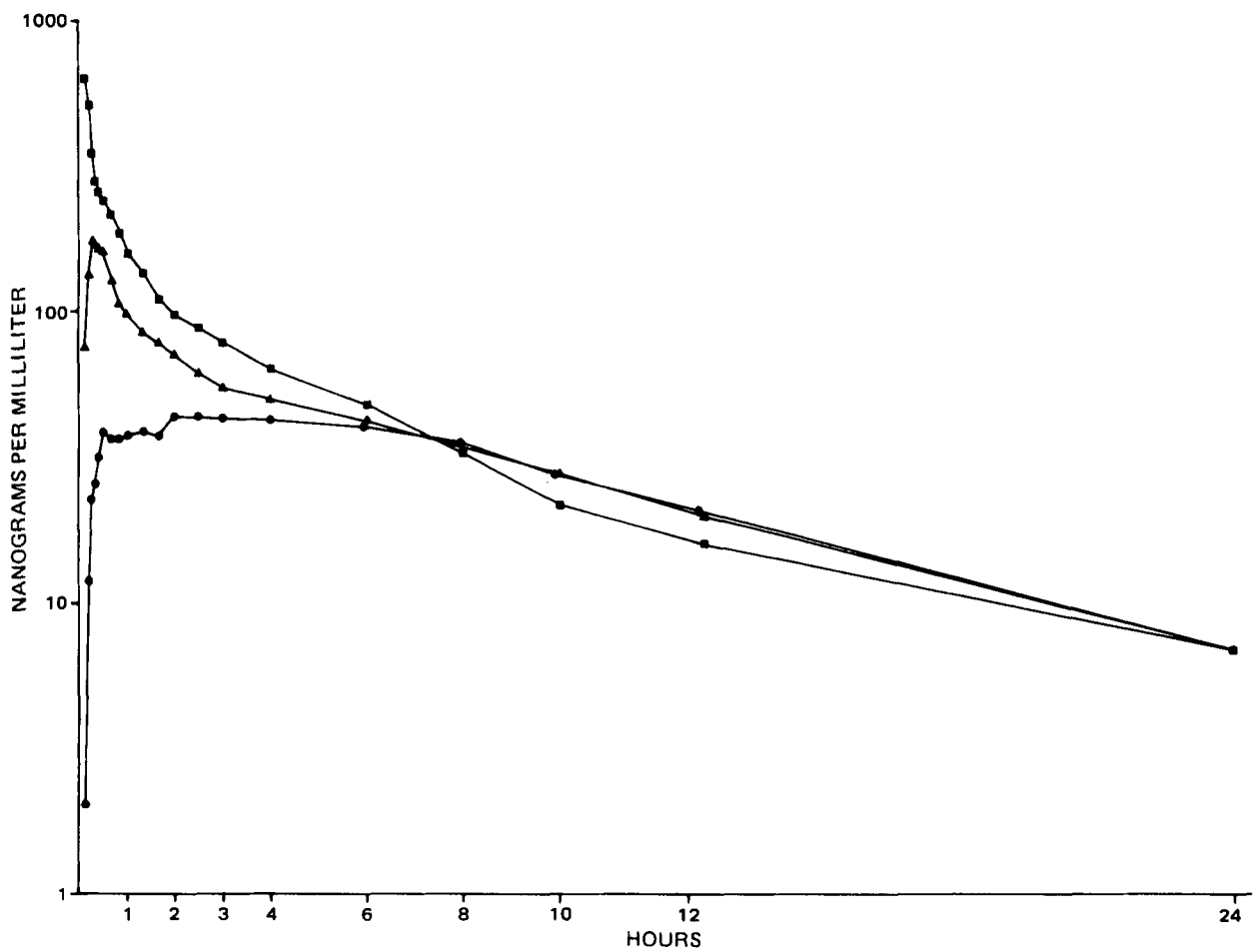

Figure 3—*Tilidine, nortilidine, and bisnortilidine concentrations in human plasma following single intravenous dose of 50 mg of tilidine hydrochloride.*

EXPERIMENTAL

Reagents and Standards—All reagents[2] were analytical grade. Aqueous solutions were prepared in double-distilled water. The following reagents and standards were used: 1 *N* NaOH, aqueous; 0.01 *N* NaOH, aqueous; 0.1 *N* HCl, aqueous; cyclohexane, distilled; sodium sulfate, anhydrous; chloroform; ethyl acetate; tilidine hydrochloride[1]; nortilidine hydrochloride[3]; bisnortilidine hydrochloride[4]; and 1[(*R*)-*N*-(dipropyl)-amino]-4-phenyl-4-ethoxycarbonylcyclohexene as the internal standard[5].

Apparatus—Measurements were carried out with a gas chromatograph equipped with a nitrogen–phosphorus detector and a 0.001–10-v recorder[6]. Retention times and peak areas were determined with a computing integrator[7]. For the sample preparations, an automatic shaker[8] and rotary evaporators[9] were used. All glassware was washed in detergent, thoroughly rinsed with water, soaked in chromic acid overnight, rinsed with water again, and dried in an oven at 70°.

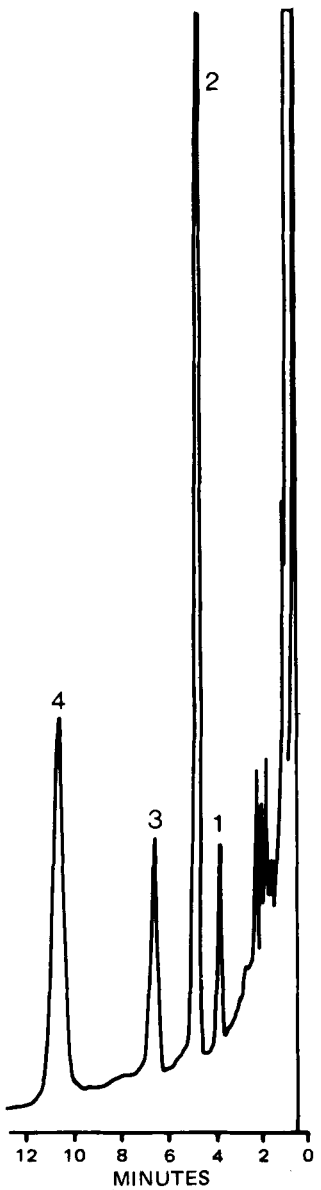

Figure 4—*Chromatogram of a plasma extract 80 min after oral administration of 50 mg of tilidine hydrochloride to a male subject. Key: 1, tilidine; 2, nortilidine; 3, bisnortilidine; and 4, internal standard. Attenuation was 8 × 1.*

GLC Conditions—The 1.8-m × 2-mm silanized glass column was packed with 1% CRS 101[10] and 1.5% LAC-4-R-886[11] on 80–100-mesh Gas Chrom Q[12]. To prepare the column packing, the stationary phases were dissolved in chloroform, poured over Gas Chrom Q, mixed well, and left to stand for ~15 min. Most of the solvent was then evaporated at 70° (without vacuum), followed by final evaporation on a rotary evaporator under vacuum at 50°. During the filling of the column, it was held in an ultrasonic bath[13] from time to time to ensure optimal packing. The column was conditioned for 24 hr at 180° prior to use.

The temperature settings were: injection port, 190°; outlet manifold, 200°; and column oven, 165°. The duo-dial knob of the temperature unit of the nitrogen detector was set at 600. The detector gases were hydrogen at 2 ml/min and synthetic air at 100 ml/min. The carrier gas was nitrogen at 20 ml/min. The recorder was set at 2-mv full-scale deflection, and the chart speed was 5 mm/min.

Procedure—Plasma or urine 1–5 ml (amount depended on the concentration), was placed in a separator. Internal standard, 500 ng, dissolved in 100 μl of double-distilled water, and 0.5 ml of 1 *N* NaOH were added. The sample was extracted three times for 2 min with 20-ml portions of cyclohexane on an automatic shaker. The combined cyclohexane solutions were washed once with 5 ml of 0.01 *N* NaOH and 5 ml of double-distilled water. Then the cyclohexane was reextracted with 10 and 5 ml of 0.1 *N* HCl.

The cyclohexane solution was discarded, and the combined hydrochloric acid solutions were evaporated to dryness on a rotary evaporator in a 25-ml round-bottom flask at 40°. The residue was transferred into a 5-ml tapered flask with 0.1 *N* HCl (3 × 1 ml) and again evaporated to dryness. The content of the flask was redissolved in 200 μl of chloroform, followed by evaporation with a gentle stream of nitrogen to concentrate the compounds in the tip of the flask.

Dry ammonia vapors were blown into the flask, and ~10–20 μl of ethyl acetate was added. The flask was stored in ice; 1–4 μl of this solution was injected into the gas chromatograph. The peak areas were calculated with a computing integrator. The computing integrator also calculated the amounts directly with previously determined stored calibration factors.

Standard Calibration Curves—Blank plasma (5 ml) was spiked with known concentrations (25–1000 ng) of aqueous solutions of tilidine, nortilidine, and bisnortilidine and with 500 ng of internal standard in 100 μl of water. The analyses were carried out as described. Standard curves were prepared by plotting the peak area ratios (substances to internal standard) *versus* nanograms of the determined substances (Table I).

RESULTS AND DISCUSSION

GLC analysis of secondary and primary amines is often hampered by the adsorption of these compounds on the GLC columns. In earlier studies, the support material (80–100-mesh Chromosorb G H.P.) was coated with 0.5% PEI and 0.75% LAC-4-R-886 to suppress column support activity. The alkaline properties of polyethyleneimine resulted in a GLC column on which nortilidine and bisnortilidine could be chromatographed over a long period without any noticeable adsorption. A number of biological samples were analyzed with this type of column.

During these experiments, a flame-ionization detector was used for GLC analysis. When a nitrogen-selective detector became available, polyethyleneimine was replaced by CRS 101 (amine-substituted silicone phase), which also possesses alkaline properties and deactivating adsorption sites. CRS 101 appeared to be a thermally stable stationary phase because of its low volatility; polyethyleneimine, in combination with the nitrogen-selective detector, showed a permanent baseline drift and extensive noise, limiting the advantages of a nitrogen-selective detector.

On the ethylene glycol succinate–CRS 101 column, the retention times of the substances to be determined were: tilidine, 3.7 min; nortilidine, 4.8 min; bisnortilidine, 6.7 min; and internal standard, 11.0 min. A typical chromatogram of standards directly injected onto the column is shown

[2] Merck, Darmstadt, West Germany.

[3] Ethyl DL-*trans*-2-(methylamino)-1-phenyl-3-cyclohexene-1-carboxylate hydrochloride. Prepared by the Chemical Department, Gödecke Research Institute, Freiburg, West Germany.

[4] Ethyl DL-*trans*-2-amino-1-phenyl-3-cyclohexene-1-carboxylate hydrochloride [isolated from rabbit urine (2)].

[5] Prepared by the Chemical Department, Gödecke Research Institute, Freiburg, West Germany.

[6] Model 900, Perkin-Elmer Corp., Überlingen, West Germany.

[7] Autolab System I, Spectraphysics GmbH, Darmstadt, West Germany.

[8] Bühler, Tübingen, West Germany.

[9] Büchi, Buchs, Switzerland.

[10] Chemical Research Services, Addison, Ill.

[11] Varian GmbH, Darmstadt, West Germany.

[12] WGA, Düsseldorf, West Germany.

[13] Bransonic 32, Branson Europe B.V., Soest, The Netherlands.

in Fig. 1. Chromatograms of a blank plasma sample (A) and a 10-ml plasma sample spiked with 50 ng of tilidine, nortilidine, and bisnortilidine and 500 ng of internal standard (B) are shown in Fig. 2.

The internal standard showed similar extraction properties to tilidine and the metabolites and was consequently well suited for quantitative analysis. The recovery of all substances from plasma ranged from 88 to 92%.

The accuracy and precision of this method are reflected by the data obtained from triplicate analyses of spiked plasma samples at different concentrations (Table I). The sensitivity limit was ~1 ng/ml for all three substances with a 5-ml sample.

The usefulness of an integrator system was checked by a special case regression analysis with the assumption that the line passes through the origin. For tilidine, nortilidine, and bisnortilidine, correlation indexes of 99.74, 99.38, and 99.90%, respectively, were found. In all three cases, the correlation coefficient was above 0.996. The slopes of the regression lines pressed through the origin did not differ from those obtained by a regression analysis with both degrees of freedom. With both methods, slopes of 0.0040, 0.0042, and 0.0029 for tilidine, nortilidine, and bisnortilidine, respectively, were calculated.

Some drugs or their metabolites were responsible for peaks appearing later in the chromatographic run and delayed the analysis. These substances must be eluted by increasing the column temperature prior to the next analysis. Therefore, volunteers who have taken no medication should be used for pharmacokinetic studies.

The described method is well suited for the determination of tilidine, nortilidine, and bisnortilidine in plasma and urine following therapeutic doses of the analgesic tilidine hydrochloride to humans. The results of a plasma level study after a single administration of 50 mg iv of tilidine hydrochloride to a fasting male volunteer (age 32, 70 kg) are given in Fig. 3. Figure 4 shows a typical chromatogram of a plasma sample after oral administration of tilidine hydrochloride to a male volunteer. This method is currently employed for routine analyses of biological samples.

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Toxic Substances Produced by *Fusarium* V: Occurrence of Zearalenone, Diacetoxyscirpenol, and T-2 Toxin in Moldy Corn Infected with *Fusarium moniliforme* Sheld.

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Received July 14, 1977, from the *Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Banaras Hindu University, Varanasi-5, India.* Accepted for publication April 27, 1978. *Department of Plant Pathology, Banaras Hindu University, Varanasi-5, India.

Abstract □ Sweet corn infected with *Fusarium moniliforme* Sheld. (CMI-IMI 204057) while growing in the fields was shown to contain zearalenone, diacetoxyscirpenol, and T-2 toxin. Assays by spectral, chemical, and biological methods established the presence of these substances, with zearalenone being the most abundant. *In vitro* cultures of the fungus also produced the three toxins.

Keyphrases □ *Fusarium moniliforme* mold—infected sweet corn and *in vitro* cultures, toxins isolated and identified □ Toxins—isolated and identified in extracts of sweet corn infected with *Fusarium moniliforme* mold and *in vitro* cultures

Toxicity of natural products from *Fusarium* has received wide attention because of its greater incidence and, perhaps, greater importance than aflatoxins (1, 2). As part of a continuing study on *Fusarium*-induced toxicity of foodstuffs (3–6), this paper reports the isolation, characterization, and quantitation of three mycotoxins, zearalenone, diacetoxyscirpenol, and T-2 toxin, from moldy sweet corn (*Zea mays* Linn., local variety) infected with *Fusarium moniliforme*[1] Sheld. while growing in the fields. The samples were collected from the valley of the Ganges in the Varanasi District of Uttar Pradesh, India, during July 1976, when they were ready for table use.

EXPERIMENTAL

Extraction of Mycotoxins—The ground moldy corn (about 500 g) was macerated in a blender[2] with chloroform (2 liters), and the mixture was kept at 35 ± 5° for 1 week. It then was filtered, and the solvent was removed from the chloroform extract when a thick brown oily liquid was obtained. This substance was dissolved in methanol–water (80:20, 100 ml) and extracted with *n*-hexane (3 × 30-ml portions) to remove the lipid materials.

The aqueous methanol layer was further diluted with water (50 ml) and then extracted with chloroform–ethyl acetate (1:1, 2 × 30-ml portions). Evaporation of the chloroform–ethyl acetate extract gave a light-brown oily substance (1.08 g), which was found to be biologically active when tested according to literature methods (7, 8). It showed several fluorescent and Ehrlich-positive spots on TLC on silica gel G[3]. Benzene–methanol–acetic acid (24:2:1) was used as the developer.

A portion of the oily liquid (0.52 g) was dissolved in chloroform (10 ml) and chromatographed over a column of silica gel[4] (60–120 mesh, 1.8 × 40 cm). Elution was carried out with benzene–ethyl acetate (80:20, 200 ml). Fractions (10 ml) were collected.

Zearalenone—The residue from fractions 4–7, an amorphous solid (8 mg), showed a faint blue fluorescent spot, R_f 0.48, under UV light and an olive-green fluorescent spot when sprayed with concentrated sulfuric acid with subsequent heat treatment (100–110° for 10 min) on thin-layer plates.

Attempts to crystallize the solid from common organic solvents were unsuccessful. The compound fragmented before giving any molecular

[1] The identity of the fungus, Strain CMI-IMI 204057, was confirmed by the Commonwealth Mycological Institute, Kew, England.

[2] Waring.

[3] E. Merck, Darmstadt, West Germany.

[4] British Drug Houses, Poole, England.

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