High-Performance Liquid Chromatographic Determination of Chlordiazepoxide and Major Related Impurities in Pharmaceuticals

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Abstract □ A rapid, precise, forward-phase (adsorption) high-performance liquid chromatographic procedure is presented for the determination of chlordiazepoxide and two common impurities, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide and 2-amino-5-chlorobenzophenone, in commercial formulations and for the determination of the benzophenone in the chlordiazepoxide drug substance. The method involves simultaneous quantitation of chlordiazepoxide and the 1,3-dihydro impurity, followed by quantitation of the benzophenone from a separate sample extract using a second mobile phase. A single microparticulate silica gel column is used throughout. Nitrazepam and *o*-dinitrobenzene are the internal standards. Quantitation is by peak area using a computing integrator, except that the peak due to the benzophenone is quantitated by peak height. The described procedure is of equivalent precision, but superior accuracy, to the BP 1973 spectrophotometric procedure for the analysis of chlordiazepoxide in chlordiazepoxide formulations. Quantitation of the 1,3-dihydro and the benzophenone impurities at levels as low as 6.3 and 0.9 ng, respectively, is demonstrated.

Keyphrases □ Chlordiazepoxide—and related impurities, high-performance liquid chromatographic analysis, commercial formulations □ High-performance liquid chromatographic analysis—chlordiazepoxide and related impurities, commercial formulations □ Tranquilizers—chlordiazepoxide and related impurities, high-performance liquid chromatographic analysis, commercial formulations

Pharmacopeial specifications for the tranquilizer chlordiazepoxide [7-chloro-2-(methylamino)-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide] and its hydrochloride salt (I) include limit tests for the impurities 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide (II) and 2-amino-5-chlorobenzophenone (III) (1–3). These specifications allow a maximum of 0.1% of II and 0.01% (1, 2) or 0.05% (3) of III in the chlordiazepoxide drug substance. Specifications for capsules containing the hydrochloride salt permit a maximum of 3.0 and 0.1% for II and III, respectively (1, 3); tablets prepared with chlordiazepoxide base may contain up to 4.0% of II and 0.1% of III (2).

Numerous procedures have been reported for the quantitation of chlordiazepoxide in pharmaceuticals and biological fluids (1–9). However, none of these methods describes the quantitation of all three compounds. Spectrophotometric methods, such as those described in the pharmacopeias (1–3), are not specific for I since II exhibits a similar absorption spectrum. GLC has been used to quantitate I (8), but attempts in this laboratory to develop a GLC analysis for I–III were unsuccessful because of poor peak shape, lack of separation of I and II, long analysis times, sample decomposition, and/or poor precision.

High-performance liquid chromatography (HPLC) has been used extensively for the analysis of drugs in pharmaceuticals (10–14), including the qualitative (15) and quantitative (16) analysis of benzodiazepines. Chlordiazepoxide, but not II or III, has been quantitatively analyzed by HPLC (17).

This report describes a rapid, two-part HPLC method for the analysis of I–III in chlordiazepoxide formulations and for the analysis of III in I drug substance.

EXPERIMENTAL

Materials—Chlordiazepoxide hydrochloride[1] [tested to conform to USP (1) specifications], nitrazepam[1], and *o*-dinitrobenzene[2] were used as received. Compound II was prepared by controlled alkaline hydrolysis of I, recrystallized from ethanol–hexane, and dried at 56° *in vacuo* (mp 231–234°). Purity was established chromatographically (TLC and HPLC) by comparison to USP reference standard material[3]. Compound III[2] was recrystallized twice from ethanol and dried *in vacuo* at 61° (mp 97–98°). Reagent grade tetrahydrofuran[4] was distilled from ferrous sulfate[4] and stored over molecular sieves[5] under nitrogen. All other solvents and reagents were analytical reagent grade, except *n*-hexane[6] which was UV grade.

Apparatus—A liquid chromatograph[7], fitted with a septumless injection port[7], a fixed wavelength UV detector[7] (254 nm), and a computing integrator[8], was used. The detector was attenuated to 0.04 absorbance unit full scale (aufs) throughout. The column (25 × 0.216 cm i.d.), packed with 5-μm diameter silica gel[9] using a balanced density slurry technique similar to that described by Majors (18), was operated at ambient temperature and a flow rate of 60 ml/hr.

Mobile Phases—For the quantitation of I and II, System A, 5% ammoniacal ethanol and 30% tetrahydrofuran in *n*-hexane, was used. Ammoniacal ethanol (10% v/v) was prepared from concentrated ammonium hydroxide and absolute ethanol.

For the quantitation of III, System B, 25% tetrahydrofuran in *n*-hexane, was used.

Mobile phases were prepared as required, degassed (reflux, 5 min), and stored in the solvent reservoirs of the instrument.

Stock Solutions—*Nitrazepam (IV) Internal Standard Solution*—Approximately 15 mg of IV, accurately weighed, was transferred to a 500-ml volumetric flask, dissolved, and brought to volume with ethyl acetate (final concentration of 30 μg/ml).

o-Dinitrobenzene (V) Internal Standard Solution—Approximately 25 mg of V, accurately weighed, was transferred to a 500-ml volumetric flask, dissolved in a minimum of ethanol, and brought to volume with *n*-hexane (final concentration of 50 μg/ml).

Standard Solution of I—Approximately 25 mg of I, accurately weighed, was transferred to a 100-ml volumetric flask, dissolved, and brought to volume with 0.1 *N* aqueous hydrochloric acid (final concentration of 250 μg/ml).

Standard Solution of II—Approximately 20 mg of II, accurately weighed, was transferred to a 200-ml volumetric flask, dissolved, and brought to volume with ethyl acetate (final concentration of 100 μg/ml).

Standard Solution of III—Approximately 20 mg of III, accurately weighed, was transferred to a 200-ml volumetric flask, dissolved, and brought to volume with *n*-hexane. Then 10.0 ml of this solution was transferred to a 50-ml volumetric flask and brought to volume with *n*-hexane (final concentration of 20 μg/ml).

[1] Hoffmann-La Roche, Montreal, Quebec, Canada.
[2] Aldrich Chemical Co., Montreal, Quebec, Canada.
[3] The United States Pharmacopeial Convention, Inc., Rockville, Md.
[4] British Drug Houses, Toronto, Ontario, Canada.
[5] Fisher Scientific Co., Ottawa, Ontario, Canada.
[6] Burdick and Jackson Laboratories, Muskegon, Mich.
[7] Model 4100, Varian Aerograph, Palo Alto, Calif.
[8] Autolab System I, Spectra-Physics, Santa Clara, Calif.
[9] LiChrosorb SI 60, Brinkmann Instruments (Canada) Ltd., Rexdale, Ontario, Canada.

All stock solutions and sample preparations were protected from light by wrapping the tubes or flasks with aluminum foil.

Standard Curves for I and II—Five standard solutions were prepared to contain between 200 and 400 μg of I/ml in 0.1 *N* aqueous hydrochloric acid. Similarly, five solutions were prepared to contain between 1.7 and 300 μg of II/ml in ethyl acetate. Ten milliliters of the highest concentration solution of I and 1.0 ml of the highest concentration solution of II were pipetted into a 30-ml screw-capped tube[10] along with 1.0 ml of concentrated ammonium hydroxide and 12.5 ml of IV internal standard solution. The other standard solutions were prepared in the corresponding manner. The five tubes were tumbled on a rotator[11] at 30 rpm for 30 min and then centrifuged[12] at 2000 rpm for 10 min. Duplicate 5-μl aliquots of the supernatant ethyl acetate layer were chromatographed using mobile phase System A.

Linearity Curve for III—Six solutions were prepared to contain between 1.0 and 35 μg of III/ml in *n*-hexane. Aliquots of 1.0 ml of each solution were pipetted into individual 5-ml volumetric flasks along with 3.0 ml of V internal standard solution. The solutions were diluted to volume with *n*-hexane, and duplicate 5-μl aliquots of each solution were chromatographed using mobile phase System B.

Daily Calibration Standards—For the determination of I and II, 10.0 ml of I standard solution, 1.0 ml of II standard solution, 1.0 ml of concentrated ammonium hydroxide, and 12.5 ml of IV internal standard solution were pipetted into a 30-ml screw-capped tube. The tube was tumbled at 30 rpm for 30 min and then centrifuged at 2000 rpm for 10 min. Duplicate 5-μl aliquots of the ethyl acetate layer were chromatographed using mobile phase System A.

For the determination of III, 15.0 ml of 0.1 *N* hydrochloric acid, 1.0 ml of III standard solution, and 3.0 ml of V internal standard solution were pipetted into a 30-ml screw-capped tube. The tube was tumbled at 30 rpm for 1 hr and then centrifuged at 2000 rpm for 10 min. Duplicate 5-μl aliquots of the *n*-hexane layer were chromatographed using mobile phase System B.

Analysis of Pharmaceuticals—*Determination of I and II*—Powdered tablet or capsule contents equivalent to 25 mg of chlordiazepoxide hydrochloride or to 20 mg of chlordiazepoxide base, accurately weighed, were transferred to a 100-ml volumetric flask along with 60 ml of 0.1 *N* hydrochloric acid. The stoppered flask was shaken[13] vigorously for 1 hr, brought to volume with 0.1 *N* hydrochloric acid, mixed well, and filtered[14] with the aid of suction.

Ten milliliters of the filtrate, 1.0 ml of ammonium hydroxide, 1.0 ml of ethyl acetate, and 12.5 ml of IV internal standard solution were transferred to a 30-ml screw-capped tube. The tube was tumbled on a rotator for 30 min at 30 rpm and centrifuged at 2000 rpm for 10 min. Duplicate 5-μl aliquots of the supernatant ethyl acetate layer were chromatographed using mobile phase System A.

Determination of III—Powdered tablets or capsule contents equivalent to 25 mg of chlordiazepoxide hydrochloride or to 20 mg of chlordiazepoxide base, accurately weighed, were transferred to a 30-ml screw-capped tube. Then 15.0 ml of 0.1 *N* hydrochloric acid, 1.0 ml of *n*-hexane, and 3.0 ml of V internal standard solution were added. The tubes were tumbled on a rotator for 1 hr at 30 rpm and centrifuged at 2000 rpm for 10 min. Duplicate 5-μl aliquots of the *n*-hexane layer were chromatographed using mobile phase System B.

Calculations—The slopes of the calibration curves, S_C, determined each time the mobile phase was replenished, were calculated from:

$$S_C = N_S C_I / N_I C_S \qquad \text{(Eq. 1)}$$

where N_S is the number of integrator counts for I or II or the peak height for III, N_I is the number of integrator counts for the internal standard, C_I is the concentration of the internal standard, and C_S is the concentration of the test compound I, II, or III.

The amount of each component found, expressed as a percentage of the label claim of chlordiazepoxide hydrochloride, was calculated from:

$$\% \text{ label claim} = \frac{N_S C_I F}{N_I S_C M_I} \qquad \text{(Eq. 2)}$$

where M_I is the theoretical weight of I injected (based on label claim), and F is a scaling factor (13,500 for I and II, 400 for III).

[10] Canadian Laboratory Supplies, Montreal, Quebec, Canada.
[11] Scientific Industries Ltd., Springfield, Mass.
[12] Model K, International Equipment Co., Needham Heights, Mass.
[13] Model 00, Burrell Corp., Pittsburgh, Pa.
[14] Whatman No. 3, Canadian Laboratory Supplies, Ottawa, Ontario, Canada.

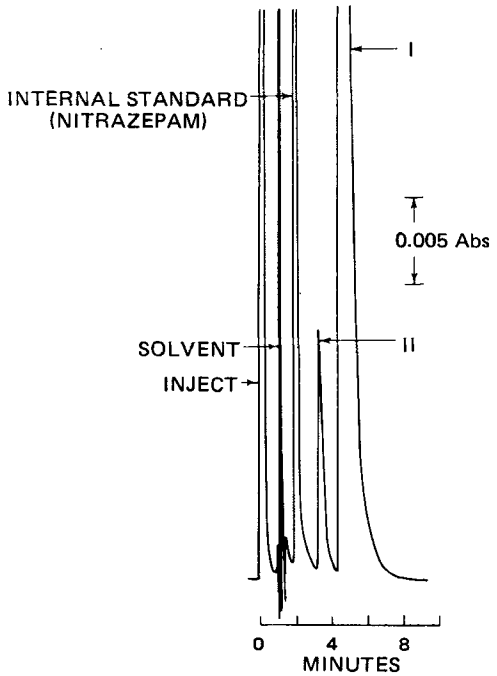

Figure 1—*High-performance liquid chromatogram of a standard solution containing 0.223 mg of chlordiazepoxide hydrochloride/ml, 7.06 μg of II/ml, and 33.0 μg of nitrazepam/ml (internal standard) using mobile phase System A.*

RESULTS AND DISCUSSION

Evaluation of Chromatographic Systems—Liquid–solid (adsorption) and bonded-phase chromatography are the modes of HPLC most applicable to the analysis of chlordiazepoxide and related impurities. Due to availability, cost, simplicity, stability, and column efficiency, liquid–solid chromatography, using a column packed with microparticulate silica gel, was chosen.

Various isocratic and gradient mobile phase systems were tried before the systems described were chosen. Attempts to find an isocratic mobile phase that eluted III behind the solvent front while eluting I and II within a reasonable time were unsuccessful because of the large relative differences in polarity among the three compounds. The inclusion of tetrahydrofuran in mobile phase System A was necessary to elute II before I. Also, III could not be detected at the low levels required (<0.01% in I) while maintaining the amount of I within the linear range of the system. The first problem was overcome by using a gradient system, but this approach had several disadvantages: lower precision, major baseline shifts during the gradient run, and longer total time due to a long column re-equilibration time. The sensitivity problem could possibly have been overcome by using a two-injection technique (overload the column with I to quantitate III and proceed normally to quantitate I and II). However, it was decided that this approach, combined with a gradient mobile phase system, would be less precise and offer little, if any, timesaving over the use of two separate isocratic systems for the analysis of III and for I and II. This latter approach allows optimum partition and chromatographic conditions to provide maximum peak size and minimum chromatograph time for all compounds.

Figure 1 shows a chromatogram obtained when the contents of a chlordiazepoxide hydrochloride capsule were analyzed for I and II using the described procedure. The internal standard, nitrazepam, and I and II were well resolved in less than 8 min. The peaks tailed somewhat, despite the basic mobile phase, but this tailing did not affect quantitation.

Figure 2 shows a chromatogram obtained when a 5-μl aliquot of a calibration standard, containing 4 μg/ml of III, was analyzed using mobile phase System B. The peaks for III and the internal standard, *o*-dinitrobenzene, were well resolved and eluted in less than 6 min.

Linearity and Standard Curves—A plot of peak area *versus* the amount of I injected was linear over the concentration range of 40–300 μg/ml[15] (0.2–1.5 μg/injection).

[15] Compound I was dissolved in 0.3% ammonium hydroxide in absolute ethanol (v/v).

Table I—Analysis of Synthetic Solutions of I and II

Sample	I			II		
	Calculated, μg/ml	Found, μg/ml	Recovery, %	Calculated, μg/ml	Found, μg/ml	Recovery, %
1	300	296	98.8	12.8	12.6	98.4
2	192	193	100.4	5.9	6.0	101.7
3	180	183	101.5	2.9	2.9	100.0
4	244	245	100.4	7.3	7.1	97.3
5	211	211	100.2	20.3	20.6	101.5
Mean recovery, %			100.3			99.8
RSD, %			±1.0			±2.6

A standard curve for I, covering the concentration range of 150–300 μg/ml (0.75–1.50 μg/injection) was linear with a slope of 1.37 ± 0.02 and a negligible intercept (0.005). By using the sample preparation procedure described, this standard curve is applicable to samples containing 80–160% of the theoretical weight of I (25 mg).

Duplicate calibration standards were prepared daily and injected periodically to determine the slope of the standard curve. The slope varied by less than 1% on any given day and by less than 3% over the study. Since the current value of the slope was experimentally determined and used in the calculations, small variations in the slope did not affect the accuracy of the procedure.

An identical procedure was followed for the determination of II. A plot of peak area *versus* the amount injected was linear to 140 μg/ml (0.7 μg/injection). A standard curve over the range of 1.2–20 μg/ml (0.006–0.1 μg/injection), the equivalent of 0.7–11% of the theoretical weight of I (25 mg), was linear with a slope of 1.10 ± 0.01 and a negligible intercept (0.0003). Duplicate calibration standards were used to check the slope of the standard curve, as for I.

A standard curve was not used for the analysis of III. Instead, linearity, partition, and calibration standard data were combined to show that the slope obtained from subsequent calibration standard data alone allowed III to be determined directly.

A plot of the ratio of peak height to internal standard counts *versus* the amount of III injected was linear between 1 and 35 ng/injection and passed through the origin. This range represented, using the sample preparation scheme described under *Experimental*, levels of III between 0.003 and 0.11% of the theoretical amount of I (25 mg).

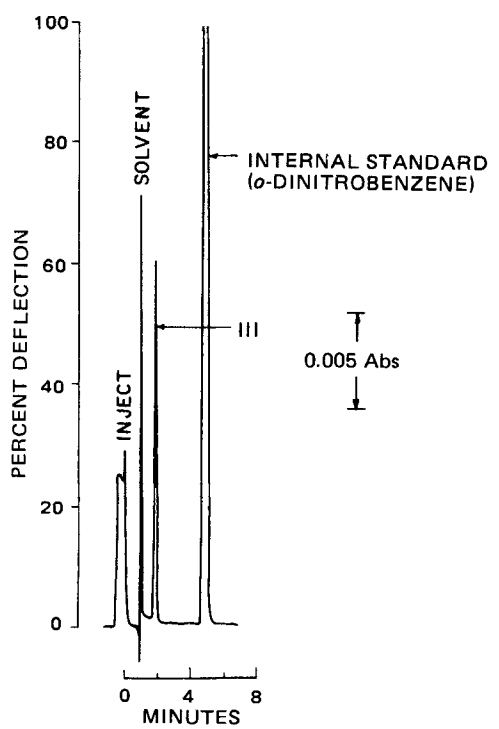

Figure 2—*High-performance liquid chromatogram of a standard solution containing 4.0 μg of III/ml and 56.0 μg of o-dinitrobenzene/ml (internal standard) using mobile phase System B.*

Sample Preparation—The times necessary for constant and complete extraction and partition of I and II from formulations were determined in two stages. Time for extraction into 0.1 *N* hydrochloric acid was determined by shaking capsule composite samples for 30, 60, and 90 min. The samples were then made alkaline and partitioned into ethyl acetate, as described, for 60 min. When the ethyl acetate layer was analyzed for I and II, levels in the samples extracted for 60 and 90 min were identical but somewhat higher than in the sample extracted for 30 min. With a 60-min extraction, the time for constant partition was determined by tumbling capsule composite samples, as described, for various times up to 60 min. No change in the peak heights for I or II were noted after 20 min.

Quantitative partition of I and II from hydrochloric acid (made basic) into ethyl acetate was demonstrated by chromatographing both layers of a capsule composite sample tumbled for 30 min. Also, when the described procedure was applied to capsule composite samples equivalent to 10, 20, and 30 mg of I, the ratio of concentrations for I and for II in the three samples was 1:2:3 in both cases.

To determine the time required to attain equilibrium in the partition of II, capsule composite samples in 0.1 *N* hydrochloric acid were tumbled with *n*-hexane, as described, for various times up to 90 min. No change in the size of the peak for III, obtained by chromatographing 5-μl aliquots of the *n*-hexane layer using mobile phase System B, was observed after 40 min.

When both layers of a calibration standard were analyzed for the internal standard (*o*-dinitrobenzene) and III, both compounds were partitioned quantitatively into the *n*-hexane layer (<99:1 concentration ratio). In addition, when the described extraction procedure was applied to capsule composite samples equivalent to 10, 20, and 30 mg of I, the concentrations of III were in the ratio 1:2:3. These data indicated complete and constant partition of III under the described conditions.

Compounds I and II did not interfere with the analysis of III since no peaks were observed for I or II when 5-μl aliquots of the *n*-hexane layer of a capsule composite sample, prepared using the procedure described for the determination of III, were chromatographed using mobile phase System A.

Quantitation—Compounds I and II were quantitated using peak area; however, quantitation of III was more precise using peak height. Since the internal standard peak was integrated in both cases, the assay for III was based on the peak height to peak area ratio and the assay for I and II was based on peak area ratios.

Reproducibility of the chromatographic system was shown by chromatographing six 5-μl aliquots of a calibration standard containing 0.19 mg of I/ml and 0.006 mg of II/ml (0.95 and 0.02 μg/injection, respectively)

Table II—Analysis of Synthetic Solutions of III

Sample	Calculated, μg/ml	Found, μg/ml	Recovery, %
1	0.179	0.178	99.6
2	0.358	0.356	99.5
3	1.79	1.83	102.1
4	4.00	3.90	97.5
5	8.10	7.90	97.5
6	16.1	16.1	100.0
7	24.9	24.8	99.6
8	32.3	31.8	98.4
Mean recovery, %			99.3
RSD, %			±1.5

Table III—Analysis of Chlordiazepoxide Formulations for I and II by HPLC and Spectrophotometric[a] Methods

Sample	Type	HPLC			Spectrophotometric, "I"[b], %
		I[b], %	II[b], %	Total[b], %	
A	Tablet, base	98.0	0.73	98.7	98.8
B	Capsule, hydrochloride	97.3	0.81	98.1	98.1
C	Capsule, hydrochloride	96.6	4.12	100.7	101.2
D	Capsule, hydrochloride	91.5	5.62	97.4	96.4
E	Tablet, hydrochloride	87.1	8.54	95.7	95.7

[a] Reference 3. [b] Percent of label claim amount of chlordiazepoxide *or* chlordiazepoxide hydrochloride.

along with the 0.03-mg/ml nitrazepam internal standard. The relative standard deviations of the peak area ratios were 0.32% for I and 2.7% for II. The analogous figure for III at a level of 0.0036 mg/ml (0.018 μg/injection) was 2.3%.

Calibration standards, stored in the dark at 4° between analyses, were analyzed for I, II, and III on each of 5 consecutive days; the relative standard deviations were 1.1, 2.7, and 2.5%, respectively. While the standards appeared to be stable for at least 1 week at 4°, fresh calibration standards were prepared daily to check the slopes of the calibration curves.

The minimum detectable amounts of II and III under the conditions described were 1 and 0.2 ng/injection, respectively. The lowest levels actually quantitated were 6.3 and 0.9 ng/injection, respectively. Under the extraction conditions described, these amounts of II and III represented 0.7 and 0.0003%, respectively, of the label claim of I.

Analysis of Solutions of Known Concentration—Five solutions of I and II, prepared in the same manner as the calibration standard solutions, were analyzed using the described procedure. Table I shows that the mean recoveries for I and II were 100.2 and 99.8% with relative standard deviations of 1.0 and 2.6%, respectively.

Similarly, eight solutions were prepared to contain known concentrations of III (0.18–32 μg/ml) in the same way as solutions used for the determination of linearity. Table II shows that the mean recovery was 99.3% with a relative standard deviation of 1.5%. For this assay, only the calibration standard used to determine the slope of the standard curve was prepared in the same way as for linearity samples. For the analysis of pharmaceuticals, the procedure described in *Experimental* was used.

Analysis of Pharmaceuticals—Five formulations of chlordiazepoxide or chlordiazepoxide hydrochloride, capsules or tablets, were analyzed by the BP spectrophotometric procedure (3) and by the described HPLC procedure for I and II. Aliquots of the same tablet or capsule composite were analyzed in each case. The spectrophotometric method is nonspecific, since both I and II exhibit absorbance maxima at 308 nm, the wavelength used in the assay. It was not surprising, therefore, that the sum of the levels of I and II determined by the HPLC procedure agreed closely with the level of I determined by the spectrophotometric method (Table III). The level of II exceeded the allowed limit in three cases; however, the samples assayed were several years old and were not representative of current formulations.

The precision of the procedure for the determination of I and II in formulations was determined by analyzing Sample B (Table III) five times. Percent relative standard deviations of 0.2 and 1.8% were measured for I and II, respectively.

Five formulations of chlordiazepoxide or chlordiazepoxide hydrochloride were analyzed for III using the described procedure (Table IV). Very low levels of III were encountered; in all cases, these levels were well within the allowable limits. Results for Formulation E (Tables III and IV) showed that a high level of II did not necessarily imply a high level of III. The other four samples listed in Table IV were selected to contain the highest levels of III from over 30 screened by the appropriate TLC limit test (1–3). The precision of the procedure for the determination of III in formulations was shown by analyzing Sample F five times. A percent relative standard deviation of 1.4% was measured.

Table IV—Analysis of Chlordiazepoxide Formulations for III by HPLC[a]

Sample	Type	III[b], %
E	Tablet, hydrochloride	0.030
F	Capsule, hydrochloride	0.015
G	Capsule, hydrochloride	0.009
H	Capsule, hydrochloride	0.006
I	Capsule, hydrochloride	0.015

[a] Using mobile phase System B. [b] Percent of label claim amount of chlordiazepoxide hydrochloride.

Five samples of chlordiazepoxide or chlordiazepoxide hydrochloride drug substance also were analyzed for III. In all cases, levels below the quantifiable limit of 0.003% were found, well below the allowable limits of 0.01%.

The described method is applicable to both tablets and capsules and both the free base and hydrochloride salt forms of chlordiazepoxide. Interference from excipients was not experienced. The method could be adapted to the analysis of single 5- or 10-mg tablets or capsules by reducing the volumes used for extraction and may be used as described for single 25-mg tablets or capsules.

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