(15) C. Highta, D. L. Mays, and M. Garofalo, *Anal. Chem.*, **43**, 1530(1971).
(16) S. Kawai and S. Hashiba, *Bunseki Kagaku*, **13**, 1223(1964).
(17) H. Vanderhaeghe, E. Evrard, and M. Claesen, in "XXIII International Congress Pharm. Soc. 1963," Sept. 1963, p. 405.
(18) G. Tortolani and M. Mazza, *J. Chromatogr.*, **86**, 139(1973).
(19) N. E. Hyslop and R. J. Milligan, *Antimicrob. Ag. Chemother.*, **5**, 617(1974).
(20) A. Bracey, *J. Pharm. Sci.*, **62**, 1695(1973).
(21) J. Konecny, E. Felber, and J. Gruner, *J. Antibiot.*, **26**, 135(1973).
(22) J. J. Nelson, *J. Chromatogr. Sci.*, **11**, 28(1973).
(23) B. B. Levine, *Arch. Biochem. Biophys.*, **93**, 50(1961).
(24) G. A. Caron, *Immunology*, **6**, 81(1963).
(25) B. B. Levine, *J. Exp. Med.*, **112**, 1131(1960).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 25, 1974, from *Control Analytical Research and Development, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication January 21, 1975.

Acknowledgment is made to Bristol Laboratories for the supply of penicilloic acid of ampicillin. The technical assistance of J. F. Baker, W. H. Tafolla, and W. Vanden Bosch also is acknowledged. The authors also thank A. R. Lewis for statistical analysis.

[x] To whom inquiries should be directed.

Determination of Major Impurity in Chlordiazepoxide Formulations and Drug Substance

W. N. FRENCH, F. F. MATSUI, and S. J. SMITH [x]

Abstract □ Procedures for quantitating the lactam impurity, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, which can be present in chlordiazepoxide formulations, is presented. The method consists of trapping chlordiazepoxide in sulfuric acid in kieselguhr, eluting the impurity with ether, and quantitating by UV spectrophotometry in absolute alcohol at 312 nm.

Keyphrases □ Chlordiazepoxide and chlordiazepoxide hydrochloride—UV analysis for lactam impurity (7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide) □ 7-Chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide—UV analysis in chlordiazepoxide and chlordiazepoxide hydrochloride □ UV spectrophotometry—analysis, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide in chlordiazepoxide formulations

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

While limit tests are effective in monitoring impurities in chlordiazepoxide drug compounds and formulations, quality assurance procedures often require that the levels of impurities present in a drug substance or formulation be known precisely. This article describes methodology employing a "trap" column suitable for the quantitation of the impurity I present in the drug substance and formulations.

EXPERIMENTAL

Apparatus and Materials—Glass columns (2.5 × 20 cm), 0.2 *N* sulfuric acid, water-washed ether (ethyl), and absolute ethanol were used.

For purified kieselguhr, soxhlet extract kieselguhr[1] with methanol (ACS grade) for 24 hr. Thoroughly dry the support material and store in a well-closed bottle.

For purified absorbent cotton, soxhlet extract absorbent cotton with methanol (ACS grade) for 24 hr. Dry thoroughly and store in well-stoppered bottles.

For the standard solution of I, dissolve sufficient I in ether so that each milliliter contains 150 μg.

Procedure—Place a pledget of purified absorbent cotton at the bottom of the column. Then place purified kieselguhr (3 g), which has been triturated with 0.2 *N* sulfuric acid (3 ml), over the cotton. Tamp the layer lightly but evenly. Triturate a second 3-g portion of purified kieselguhr with 3 ml of 0.2 *N* sulfuric acid, add an accurately weighed portion of powdered tablet or capsule material or drug substance equivalent to about 25 mg of chlordiazepoxide (base or salt) or 5.0 ml of standard solution of I, and mix thoroughly. Transfer this material to the prepared column and tamp into a uniform second layer. Wipe the beaker and mixing rod with purified absorbent cotton and place the cotton on top of the prepared kieselguhr column.

Elute the column with water-washed ether. Collect at least 25 ml of eluate in a 50-ml volumetric flask and evaporate the eluate to dryness, employing a stream of dry nitrogen. Dissolve the resultant residue in ethanol and make to volume with this solvent. Measure the absorbances of standard and sample solutions at 312 nm against a similarly prepared blank containing no sample or standard.

Calculate the percent of I as follows:

$$\%\,\mathrm{I} = \frac{\text{weight of I in standard } (\mu g)}{} \times \frac{A_{spl}}{A_{std}} \times \frac{100}{\text{weight of chlordiazepoxide taken } (\mu g)} \quad \text{(Eq. 1)}$$

[1] E. M. Chemicals Inc., Elmsford, NY 10523

Table I—Trap Chromatography of Chlordiazepoxide and Compound I

Sample	Chlordiazepoxide Hydrochloride Introduced to Column[a], mg	Compound I Introduced to Column[b], μg	Absorbance of Eluant at 312 nm
1	25.60	—	0.002
2	30.73	—	0.009
3	50.00	264	0.160
4	40.62	264	0.164
5	30.88	264	0.161
6	—	264	0.158
7	—	264	0.158
8	—	264	0.160

[a] Actual quantities of chlordiazepoxide weighed and added to acidic kieselguhr layer. [b] Where indicated, an ethereal solution containing 264 μg of I was added to the acidic kieselguhr layer.

where A_{spl} is the absorbance at 312 nm of the sample solution, and A_{std} is the absorbance at 312 nm of the standard solution.

DISCUSSION

When I is present in chlordiazepoxide, it cannot be quantitated by direct UV spectrophotometry because of the similar maxima exhibited by the two compounds. GLC also is unsuitable since preliminary studies indicated, as reported previously (4), that chlordiazepoxide decomposed during chromatography. Thus it was decided to take advantage of the basic nature of chlordiazepoxide and the acidic character of I and to separate the compounds by employing a trap chromatography procedure (5–7).

Since the procedure was required specifically for the quantitation of I, experimental conditions were chosen so that chlordiazepoxide would be retained on the column, thus allowing the isolation and subsequent quantitation of the I present. Sulfuric acid in the trap layer was used to ensure that all parent drug was converted to the salt form, which is insoluble in ether. The presence of the acid also ensured that no basic material, which might be present in the sample, could cause retention of I. Ether was chosen as the eluant because of its known poor solvolytic power for chlordiazepoxide and salts but good affinity for I. In addition, this solvent did not elute sulfuric acid from the trap layer under the experimental conditions.

Samples of chlordiazepoxide, of I, and of mixtures were chromatographed through trap columns, employing the procedure described. Results (Table I) indicated that chlordiazepoxide was quantitatively retained while I was recovered from eluates. Weak absorbances observed with chlordiazepoxide samples (Samples 1 and 2) and slightly higher absorbances observed with drug–I mixtures (Samples 3–5) compared to pure I (Samples 6–9) were probably due to a combination of traces of I present in the drug compound, solvent residues (discussed later), and instrumental variation. High-pressure liquid chromatography (HPLC) of trap column eluates from the mixtures (Samples 3–5) did not detect the presence of chlordiazepoxide.

Table II—Recovery of Lactam Impurity I through the Trap Column

Theoretical Concentration in Final Ethanol Solution, g/ml	Absorptivity at 312 nm Obtained with No Column Trap	Absorptivity at 312 nm after Trap Chromatography	Recovery through Column, %
30.76	36.4	35.5	97.5
21.53	35.2	35.4	100.5
15.38	35.8	35.3	98.6
6.152	35.7	36.0	100.8
4.306	34.8	35.0	100.5
3.076	35.1	34.4	98.0
Mean value	35.5[a]	35.3	99.6
Coefficient of variation	1.6	1.5	1.2

[a] Absorptivity of ethanolic solutions of impurity I measured at 312 nm was 35.6 (two determinations).

Table III—Trap Column Analysis of I in a Triturate and a Commercial Capsule Formulation

Triturate[a]			
Triturate Taken, mg	I Determined, %	Recovery, %	Capsule Composite[b], I Found, %
42.47	1.58	103.9	2.93
49.64	1.55	101.9	2.91
61.02	1.47	96.9	2.85
81.41	1.44	94.7	2.88
50.64	1.59	104.3	2.83
51.11	1.55	101.8	—
Mean recovery		100.6	2.88
Coefficient of variation		3.9	1.5

[a] Content of I, 1.52%, was determined by tumbling seven weighed aliquots of triturate with ether (25 ml) for 1 hr, filtering the mixture, washing the filter and residue with ether (3 × 5 ml), evaporating the ether solution to dryness with dry nitrogen, dissolving the residue in 50.0 ml ethanol, and measuring the absorbance of the final solution. [b] Various weights of the composite were taken for analysis.

To determine whether any I was retained by the trap chromatography column, 5-ml aliquots of various concentrations of I in ether were treated as described in *Procedure* and the absorptivities of eluants were determined. These data were compared with absorptivities obtained when similar aliquots of I were diluted to 25 ml with ether, the ether solution was evaporated to dryness with dry nitrogen, and the residue was made to 50.0 ml with ethanol. The data (Table II) indicate that I was not retained on the trap column.

The data also show that the absorbance of solutions of I is linear over the range indicated with a mean recovery of 99.68% [coeffi-

Table IV—Comparative Analysis of Compound I in Chlordiazepoxide Formulations

Sample and Form of Chlordiazepoxide	Percent I Relative to Chlordiazepoxide Found by: Trap Chromatography Method	TLC[a]	HPLC[b]
A, capsule, hydrochloride salt	5.52	5	—
B, capsule, hydrochloride salt	0.31	<0.3	0.24
C, capsule, hydrochloride salt	4.25	4	4.01
D, capsule, hydrochloride salt	4.55	5.5	—
E, capsule, hydrochloride salt	2.56	2–2.5	—
F, capsule, hydrochloride salt	3.15	3.5	—
G, capsule, hydrochloride salt	5.92	5	5.57
H, tablet, free base	0.45	0.4	0.38
I, tablet, free base[c]	8.56	8	8.49

[a] TLC was carried out on silica gel F60-precoated layers (Merck) in ethyl acetate–ethanol (9:1) (8). Visualization was effected by spraying with Bratton–Marshall reagent (1), and estimation was made by comparison with standard spots. [b] HPLC was performed in a 5-μm microparticulate silica gel column with 5% ammoniacal ethanol (3% v/v), 30% tetrahydrofuran, and 65% *n*-hexane as solvent in a Varian model 4100 HLSL liquid chromatograph with a 254-nm UV detector. The internal standard was nitrazepam. [c] A pink material in the tablet coating interfered with the trap column method. Consequently, powdered tablet containing an equivalent of 25 mg of drug was extracted with acetone, the extract was taken to dryness, the residue was taken up in the kieselguhr containing sulfuric acid, and the analysis was concluded in the manner described.

cient of variation (*CV*) 1.2%]. Manipulation of samples does not lead to the loss of material, since absorptivity measurements of a directly prepared 5.1-μg/ml solution of I in ethanol afforded an absorptivity of 35.6.

The similar absorptivity values obtained between solutions of I prepared directly in ethanol and those prepared through an ether stage indicate that any residue from ether that could be present does not appear to make a significant absorbance contribution at 312 nm. To confirm this finding, 25-ml portions of ether were evaporated to dryness with a stream of dry nitrogen, any residue present was dissolved in 50.0 ml of ethanol, and the absorbance was measured. Values of 0.01 or lower were obtained.

Results from TLC examination of chlordiazepoxide formulations carried out over an extended period showed that all formulations contained well under 0.1% 2-amino-5-chlorobenzophenone (II). Thus, normally encountered levels of II in chlordiazepoxide formulations, while present in column eluates, do not interfere significantly with the absorbance of I at 312 nm. The absorbance maximums of a solution of I in ethanol (equivalent to 3% I using procedures described under *Experimental*) and a I–II mixture (equivalent to 3 and 1%, respectively) were nearly the same, 0.531 and 0.538, respectively. The level of II utilized was 10 times higher than pharmacopeial specifications (1–3) and was present in a much greater proportion relative to I than is ever encountered in the practical situation. Thus, it can be concluded that the absorbance contribution due to II, if present, will be well within experimental variation.

The accuracy of the proposed method for quantitating I in chlordiazepoxide was determined by analyzing aliquots of lactose triturate of I. The data obtained from this study (Table III) indicated a through-column recovery of 100.58% I (*CV* 3.9%). The precision of the proposed method was determined by analyzing replicate samples of a commerical capsule formulation, which indicated a coefficient of variation of 1.5% (Table III). This coefficient of variation of 1.5% compared very closely with coefficient of variation values obtained from ethereal solutions of I measured directly (1.6) and put through the trap column (1.5) (Table II) and with results obtained when mixtures of chlordiazepoxide and I were passed through the trap column (Table I, Samples 3–5, coefficient of variation of absorbance at 312 nm, 1.28%). In comparison, it would appear that the higher coefficient of variation values obtained from I–lactose triturate samples (Table III) reflect problems encountered in attempting to obtain a homogeneous triturate of a low level of I rather than the imprecision of the method.

Nine samples of chlordiazepoxide tablets and capsules, from a number of suppliers, were analyzed for I, employing the trap column method. The results from duplicate analyses were compared with values for I obtained from TLC and/or HPLC. Data obtained (Table IV) confirm the accuracy of the method of analysis and underline the suitability of the trap column procedure for the quantitation of decomposition product I in chlordiazepoxide.

REFERENCES

(1) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, pp. 113, 114.

(2) "The National Formulary," 13th ed., Mack Publishing Co., Easton, Pa., 1970, pp. 148, 149.

(3) "British Pharmacopoeia," Her Majesty's Stationery Office, London, England, 1973, pp. 96, 97.

(4) C. McMartin and H. V. Street, *J. Chromatogr.*, **22**, 274(1966).

(5) J. Levine and J. D. Weber, *J. Pharm. Sci.*, **57**, 631(1968).

(6) *Ibid.*, **54**, 636(1965).

(7) D. J. Smith, *J. Ass. Offic. Anal. Chem.*, **49**, 536(1966).

(8) H. D. Beckstead and S. J. Smith, *Arzneim.-Forsch.*, **18**, 529(1968).

ACKNOWLEDGMENTS AND ADDRESSES

Received September 16, 1974, from the *Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada, Ottawa, Canada K1A 0L2*

Accepted for publication January 21, 1975.

The authors acknowledge the assistance of technologists in the Analytical Chemistry Section in performing analyses and thank Mr. A. G. Butterfield and Mr. R. Sears for HPLC analyses.

[x] To whom inquiries should be directed.

Spectrophotometric Determination of Acetaminophen and Dichloralantipyrine in Capsules

FOTIOS M. PLAKOGIANNIS [x] and AHMED M. SAAD

Abstract □ A rapid method for the routine determination of acetaminophen and dichloralantipyrine in capsules is reported. The determination of acetaminophen is based on the ability of its hydrolytic product, *p*-aminophenol, to produce an intensive yellow color with vanillin. The determination of dichloralantipyrine is based on the fact that it, as well as its major metabolite chloral hydrate, produces a blue color with quinaldine ethiodide. No interferences were encountered, and good recovery and precision data were obtained.

Keyphrases □ Acetaminophen–dichloralantipyrine—spectrophotometric analysis of capsule formulation □ Dichloralantipyrine–acetaminophen—spectrophotometric analysis of capsule formulation □ UV spectrophotometry—analysis, acetaminophen and dichloralantipyrine in capsules

Several methods for the quantitative determination of acetaminophen in pharmaceutical preparations are available. Most of them are colorimetric and require the hydrolysis of acetaminophen to *p*-aminophenol (1–6). A significant contribution was provided by Vaughn (6), who capitalized on the fact that acetaminophen is readily hydrolyzed to *p*-aminophenol, which produces a stable yellow color with vanillin.

Archer and Haugar (7) found out that the addition compound, dichloralantipyrine, produces, upon the addition of quinaldine ethiodide, a blue color in proportion to its chloral hydrate content.