Formation of Thermally Stable Derivatives of Chlordiazepoxide and Its Desmethyl Metabolite for GLC

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Abstract □ A derivatization procedure is described for the GLC determination of subnanogram amounts of chlordiazepoxide and nanogram amounts of *N*-desmethylchlordiazepoxide. Treatment with acetic anhydride at elevated temperature eliminates the highly polar and unstable nitrone group of these compounds by rearrangement and acetylation. The mass spectrometric fragmentation pattern of the acetyl derivatives is recorded and interpreted.

Keyphrases □ Chlordiazepoxide—and desmethyl metabolite, derivatization and GLC analyses, bulk drug □ GLC—analyses, chlordiazepoxide and desmethyl metabolite, bulk drug □ Sedatives—chlordiazepoxide and desmethyl metabolite, derivatization and GLC analyses, bulk drug

Although chlordiazepoxide has been used, and sometimes even abused, for many years for the treatment of anxiety, the analytical procedures for its determination in biological material are sometimes unsatisfactory for forensic purposes with respect to practicability, specificity, and/or sensitivity.

BACKGROUND

After publication of a colorimetric assay employing the Bratton–Marshall chromophore (1, 2), several spectrometric procedures were described for the determination of chlordiazepoxide in body fluids with (3, 4) and without (3, 5–8) prior hydrolytic cleavage to the benzophenone derivative. Due to the limited sensitivity of these spectrometric methods, a spectrofluorometric assay was developed (9) and modified (10) for use in pharmacokinetic investigations. Another fluorometric procedure, using fluorophore formation with fluorescamine after acid hydrolysis of chlordiazepoxide, is not sufficiently sensitive for the analysis of biological samples after single-dose administration (11).

Based on several investigations (12, 13), dc polarographic methods were developed for toxicological purposes (14, 15). Later (16), differential pulse polarography was used to measure therapeutic plasma concentrations. A highly sensitive radioimmunoassay (17) also was reported for chlordiazepoxide. Recently (18), benzodiazepines, including chlordiazepoxide, were determined in body fluids by high-pressure liquid chromatography, but the lower detection limit was only 5 μg/ml.

With respect to sensitivity, practicability, specificity, versatility, and general availability to forensic laboratories, GLC is the procedure most suited for identification and quantitation of chlordiazepoxide in biological material. A GLC method for its determination after acid hydrolysis was described (19), but the method lacks specificity because of the measurement of the benzophenone derivative, which also is formed from other benzodiazepines and their metabolites. Several workers also reported GLC data for chlordiazepoxide (20–22), and two procedures for its determination in serum were published (23, 24).

On the other hand, Van der Kleijn *et al.* (25) were not able to measure chlordiazepoxide by GLC, and McMartin and Street (26) observed two GLC peaks for this compound, presumably due to decomposition. Sadée and Van der Kleijn (27) stated that N^4-oxides of benzodiazepines are thermally labile under GLC conditions. Butterfield *et al.* (28) and de Silva *et al.* (29) thoroughly investigated the analysis of benzodiazepines and stated that GLC of chlordiazepoxide does not yield reproducible results. Since similar difficulties were observed in this laboratory, a procedure for derivatization of chlordiazepoxide is presented, enabling the determination of nanogram amounts by GLC.

EXPERIMENTAL

A 100-μl aliquot of freshly prepared methanolic chlordiazepoxide hydrochloride (10–10,000 ng/ml) was evaporated to dryness under a nitrogen stream. Approximately 200 μl of acetic anhydride (p.a. grade) was then added; after thorough mixing, the solution was heated at 100° for 5–10 min. The reagent was removed at room temperature under a nitrogen stream, and the residue was dissolved in an appropriate amount of methanol or hexane.

The gas chromatograph was equipped with ^{63}Ni-electron-capture and nitrogen-sensitive detectors (coiled glass tubes, 180 × 0.3 cm). The stationary phase was 3% SE-30 on 80–100-mesh Chromosorb W/AW-DMCS, the carrier gas was 40 ml of helium/min, and the electron-capture detector purge gas was 70 ml of argon–methane (95:5)/min. The column temperature was 255°, the injection port temperature was 270°, the detector temperature was 310°, and the retention time was 4 min.

Mass spectra were obtained using a gas chromatograph–mass spectrometer with electron-impact ionization.

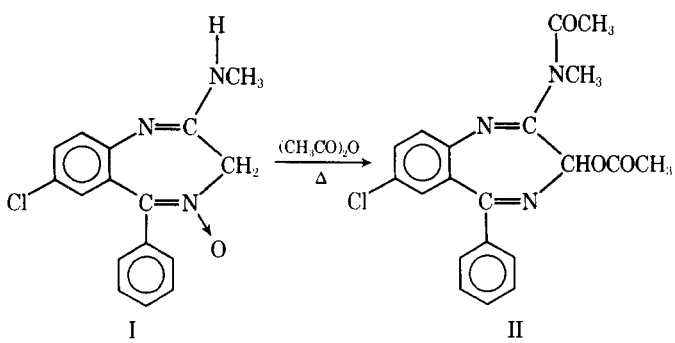
RESULTS AND DISCUSSION

It can be assumed that the unsatisfactory GLC characteristics of chlordiazepoxide (I) stem from the N^4-oxide group with its thermal instability (27) and high polarity. Therefore, a suitable derivatization procedure should eliminate the nitrone structure. Two procedures were investigated: (*a*) reduction of the nitrone with phosphorus trichloride (30) and (*b*) combined acetylation and rearrangement by treatment with acetic anhydride at elevated temperature (31, 32) as shown in Scheme I. The latter reaction, which was also proposed by Sadée and van der Kleijn (27) for GLC of *N*-methyldemoxepam (without further details being given), yielded satisfactory results and was systematically investigated.

To establish optimal conditions for derivatization, 100, 200, 500, and 1000 ng of I were treated with acetic anhydride (~200 μl) at 100° for 2, 5, 10, 15, 20, and 30 min. The reaction mixture was evaporated to dryness at room temperature under a stream of nitrogen, and the residue was dissolved in 50 or 100 μl of methanol or hexane for GLC. Optimal yields of the acetylated derivatives were obtained for reaction times of 5–10 min, but virtually all of the material was decomposed after 30 min. Formation of the acetylated product was not observed after treatment of I with acetic anhydride at room temperature for 10–60 min.

For structure elucidation, the acetylated product was subjected to GLC–mass spectrometry. The following fragmentation pattern was found: *m/e* 323 (100%, II − CH_3COOH) and 324 (91%, II − CH_3COO) and the corresponding isotope peaks at *m/e* 325 (52%) and 326 (36%). Further fragmentation was negligible, and the M^+ peak was not observed for electron energies varying between 7 and 70 ev.

The lower detection limit for GLC of I after acetylation (SE-30, helium carrier gas, electron-capture detection) was better than 50 pg; under the same conditions without prior derivatization, only 1 ng of I could be detected. Without derivatization, I exhibited broad and unreproducible peaks with helium as the carrier gas. With nitrogen or argon–methane



I II

Scheme I

0022-3549/78/0800-1176$01.00/0

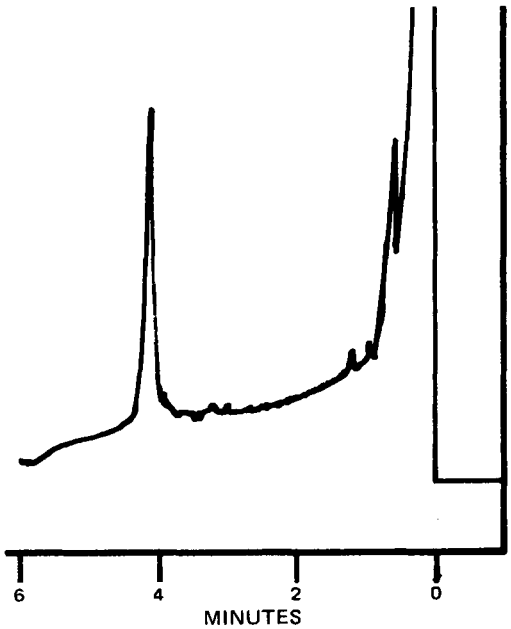


Figure 1—*Gas chromatogram of chlordiazepoxide (100 pg) after acetylation.*

(95:5), GLC was nearly impossible. In contrast, after acetylation, sharp GLC peaks were obtained with helium (Fig. 1) and the use of nitrogen or argon–methane (95:5) led only to a slight peak broadening and an insignificant decrease of sensitivity.

Fifteen samples containing 200 ng of I were acetylated as described. The coefficient of variation of the GLC peaks was ±6.2%; an internal standard was not used. The GLC responses strictly paralleled the amounts of I when 50–1500 ng of I was subjected to GLC after derivatization.

The derivatization procedure described also was applied successfully to *N*-desmethylchlordiazepoxide but not to demoxepam, the corresponding lactam. The lower detection limit for *N*-desmethylchlordiazepoxide after acetylation was about 0.5 ng when subjected to the same GLC conditions as the acetylated derivative of I. The following fragmentation pattern was observed for the diacetyl derivative of *N*-desmethylchlordiazepoxide by GLC–mass spectrometry (electron energies of 20 and 70 ev): *m/e* 369 (M^+, 24%), 327 ($M^+ - CH_2CO$, 23%), 309 ($M^+ - CH_3COOH$, 42%), 298 ($M^+ - CH_2CO - HCO$, 100%), 284 ($M^+ - CH_2CO - CH_3CO$, 57%), 256 ($M^+ - CH_2CO - CH_3CO - CO$, 100%), and 239 (55%). All of these peaks exhibited isotope peaks because of the chlorine atom. This fragmentation pattern corresponds to the ones described by Sadée (33) for 3-acetoxy derivatives of 1,4-benzodiazepin-2-ones but is in contrast to that of I after acetylation.

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