THE APPLICATION OF HPLC TO THE DETERMINATION OF SOME 1,4-BENZODIAZEPINES AND THEIR METABOLITES IN PLASMA

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- 1 A high pressure liquid chromatographic (HPLC) assay has been developed for the measurement of chlordiazepoxide and its metabolites, N-desmethylchlordiazepoxide and demoxepam in μg concentrations in human plasma.
- 2 Another metabolite was detected in appreciable amounts in the plasma from patients receiving high doses of chlordiazepoxide after the first 3 days of treatment.
- 3 Preliminary studies would indicate that this metabolite is N-desmethyldiazepam.
- 4 A modified HPLC system, using a microparticulate column was developed to separate N-desmethyldiazepam from chlordiazepoxide and its other metabolites in plasma.

Introduction

High pressure liquid chromatography (HPLC) has not been used extensively to assay 1,4-benzodiazepines, including chlordiazepoxide, in biological fluids. Scott & Bommer (1970) with a Durapak OPN column separated pure samples of diazepam, oxazepam and ten other benzodiazepines. HPLC systems have been described for the determination of partition coefficients of some 1,4-benzodiazepines (Hulshoff & Perrin, 1976). Recently, Bugge (1976) published an HPLC method for the analysis of diazepam and its N-desmethyl metabolite, using a microparticulate silica column, on which they achieved a detection limit of 25 ng ml^{-1} for both compounds. Uihlein & Hajdu (1977) reported an HPLC system for a pharmacokinetic study of the 1,4-benzodiazepine, fosazepam and its main metabolite. Small amounts of 1,4-benzodiazepines have been determined by spectrofluorimetry, polarography, gas-liquid chromatography and thin-layer chromatography, all of which, along with other techniques, have been reviewed by Clifford & Smyth (1974) and Hailey (1974).

HPLC combines many of the advantages of other methods by providing adequate separation of

components at room temperature with quantification of the drug and metabolites. Since some 1,4-benzodiazepines are thermally labile, operation at room temperature is particularly advantageous (Frigerio, Baker & Belvedere, 1973).

This report, which in part was presented at the 8th International Symposium on Chromatography and Electrophoresis in Brussels in 1975, describes a specific method for the identification and quantification of chlordiazepoxide and its metabolites (Figure 1) in biological fluids.

Methods

Chemical and reference solutions

Chlordiazepoxide hydrochloride, N-desmethylchlordiazepoxide, demoxepam, diazepam, N-desmethyl-diazepam, nitrazepam and oxazepam were provided by Roche Products Ltd, (Welwyn Garden City, Herts, England). Acetonitrile and n-hexane were spectroscopic grade, and diethyl ether and dioxane were AnalaR grade.

Aqueous solutions of the 1,4-benzodiazepines were freshly prepared, each at a concentration of $2 \mu\text{g ml}^{-1}$, except for diazepam at $4 \mu\text{g ml}^{-1}$.

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Figure 1 Structures of the 1,4-benzodiazepines.

Table 1 The recoveries of some 1,4-benzodiazepines from plasma

Compound	Extraction yield (%)
Chlordiazepoxide	97 ± 5.0 (n=7)
N-desmethy-chlordiazepoxide	99 ± 5.0 (n=7)
Demoxepam	3.8 ± 0.3 (n=3)
Diazepam	91 ± 4.4 (n=5)
Nitrazepam	8.2 ± 0.5 (n=3)

Extraction procedure

Plasma (1ml) was mixed with diazepam or nitrazepam solution (0.5 ml), deionized water (1.5 ml) and sodium hydroxide solution 0.1 M (1 ml) in a stoppered test tube (15 ml). For the calibration standards the water was replaced with a solution of chlordiazepoxide and its metabolites. The mixture was extracted with diethyl ether (5 ml) by mechanically shaking for 15 min. The resultant mixture was centrifuged, and an aliquot of the ether phase (4 ml) transferred to a tapered test tube and the ether evaporated off in a water bath at 30°C, under a stream of oxygen-free nitrogen. The residue was reconstituted with the mobile phase (100 µl) used for the HPLC analysis. The test tube was vortex mixed and aliquots (20 µl) were injected on to the column. All analyses were done in duplicate.

High pressure liquid chromatography

HPLC analyses were performed on either a Waters Associates Model ALC/GPC 501 Liquid Chromatograph or a Spectra-Physics Model 3500B Liquid Chromatograph, with UV detection at 254 nm. Three columns were used, viz.

1. A column (600 × 2.3 mm, i.d., stainless steel) dry packed with Bondapak C₁₈/Corasil, 35–52 µm (Waters Associates). The mobile phase consisted of an acetonitrile : aqueous ammonium carbonate (0.1%), 30 : 70 v/v mixture (flow rate 1.0 ml min⁻¹).
2. A commercial column (100 × 4.6 mm i.d., stainless steel) that had been packed with 5 µm ODS-Spherisorb (Phase Separations). The mobile phase was the same as in Column 1, 28 : 72 v/v mixture (flow rate 0.8 ml min⁻¹).
3. A column (450 × 2.3 mm, i.d., stainless steel) dry packed with Vydac adsorbent (Chromatronix). The mobile phase consisted of 9% dioxan in n-hexane (flow rate 1.0 ml min⁻¹).

Results

Figure 2 is a typical chromatogram, illustrating the separation of demoxepam (IV), N-desmethy-chlordiazepoxide (II), chlordiazepoxide (I) and diazepam (V), internal standard, obtained from plasma extracts on Column 1. A linear relationship between the ratios of chlordiazepoxide (or metabolite) and diazepam was obtained over the concentration range studied (Figure 3). Under the conditions described the extraction yields are shown in Table 1. By doubling the length of Column 1 it was possible to improve the resolution of a peak appearing as a shoulder on the chlordiazepoxide peak in ether

Figure 2 Chromatograms of plasma samples from chronic alcoholics receiving chlordiazepoxide orally (a) Control extract; (b) Test extract. Conditions: Column (600 × 2.3 mm i.d.) Bondapak C₁₈/Corasil; 35–52 µm; mobile phase, acetonitrile: 0.1% aqueous ammonium carbonate (30:70 v/v); flow rate 1.0 ml min⁻¹; ambient temperature. Peaks IV = demoxepam, II = desmethylchlordiazepoxide, I = chlordiazepoxide, V = diazepam.

Figure 3 Calibration curves for the determination of chlordiazepoxide (▲) and desmethylchlordiazepoxide (■) in plasma; each point is either the mean of three chlordiazepoxide/diazepam peak ratios or three desmethylchlordiazepoxide/diazepam peak ratios, with its s.d.

extracts from patients who had been receiving high doses of chlordiazepoxide for at least 3 days (Figure 4). Using a silica column, this metabolite could be resolved from chlordiazepoxide and its other metabolites (Figure 5). The microparticulate ODS-column (II) gave a marked improvement in resolution, since demoxepam (IV), nitrazepam (VI, internal standard), N-desmethylchlordiazepoxide (II), chlordiazepoxide (I) and N-desmethyldiazepam (III) are well resolved from each other (Figure 6).

Discussion

In planning a pharmacokinetic study of chlordiazepoxide in chronic alcoholics it was considered that any assay technique should be sensitive, rapid and yield information about known metabolites. High pressure liquid chromatography was thought to be ideal if a system could be found that would resolve chlordiazepoxide from its two major metabolites, N-desmethylchlordiazepoxide and demoxepam.

Modification of a procedure described in a 'Waters' Application Brief gave good resolution of the 1,4-benzodiazepines on Column I (Figure 2). The single

Figure 4 Chromatograms of (a) 1,4-benzodiazepines recovered from plasma; (b) an ether extract of plasma from a patient receiving multiple doses of chlordiazepoxide. Conditions: Column (1200 × 2.3 mm i.d.) Bondapak C₁₈/Corasil35–52 μm; mobile phase as Figure 2; flow rate 0.8 ml min⁻¹; ambient temperature. Peaks IV = demoxepam, VII = oxazepam, II = desmethylchlordiazepoxide, III = desmethyl diazepam, I = chlordiazepoxide, V = diazepam.

extraction procedure usually resulted in an extract which was free from interfering peaks (Figure 2); if any appeared in the control they were subtracted from the test sample. Since the peaks were fairly symmetrical and resolved, peak heights were used as a measure of concentration. The extraction procedure was satisfactory for all the 1,4-benzodiazepines examined except demoxepam (Table 1). It is known

Figure 5 Chromatograms of (a) an ether extract of plasma from a patient receiving multiple doses of chlordiazepoxide; (b) desmethyldiazepam and diazepam, internal standard recovered from plasma. Conditions: Column (450 × 2.3 mm i.d.) dry packed with Vydac adsorbent; mobile phase 9% dioxan in n-hexane; flow rate 1.0 ml min⁻¹; ambient temperature. Peaks V = diazepam and III = desmethyldiazepam.

(Koechlin, Schwartz, Krol & Oberhansli, 1965) that under alkaline conditions demoxepam is readily hydrolyzed to the 'open lactam' and this would explain the low extraction yields for demoxepam.

Column I was used for examining the plasma levels of chlordiazepoxide and its metabolites in chronic alcoholics (Whiting, Lawrence, Meier & Skellern, 1978). At day three of this study, it was

Figure 6 Chromatograms of (a) a plasma blank; (b) 1,4-benzodiazepines recovered from plasma. Conditions: Column (100 × 4.6 mm i.d.) packed with 5 µm ODS-Spherisorb; mobile phase acetonitrile: 0.1% aqueous ammonium carbonate, 28:72 v/v; mixture; flow rate 1.0 ml min⁻¹; ambient temperature. Peaks IV=demoxepam, VI=nitrazepam, II=desmethylchlordiazepoxide, I=chlordiazepoxide, III=desmethyldiazepam.

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(Received June 16, 1977)