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Note

Quantitative determination of pinazepam and its metabolites in blood and urine by high-performance liquid chromatography

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The *in vivo* study of the kinetics and biotransformation of pinazepam (7-chloro-5-phenyl-1-propargyl-1,4-benzodiazepin-2-one) by electron-capture gas–liquid chromatography has been described previously[1]. N-Depropargylation and C-hydroxylation at position 3 were shown to be the major metabolic pathways of pinazepam (Fig. 1).

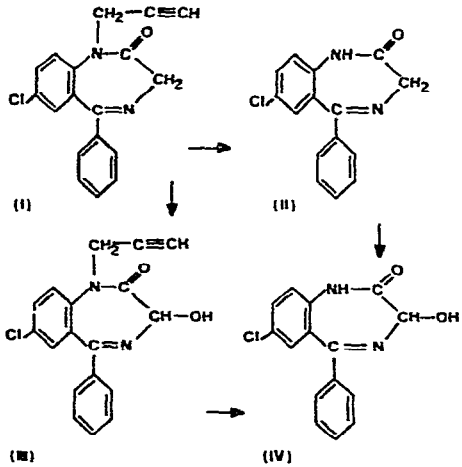

Fig. 1. The metabolism of pinazepam. I = Pinazepam; II = desmethyldiazepam; III = 3-hydroxypinazepam; IV = oxazepam.

This paper describes the recent development of another sensitive and reproducible method, based on high-performance liquid chromatography (HPLC).

EXPERIMENTAL AND RESULTS

Reagents and standards

The reagents used were hydrochloric acid R.S. and sodium hydroxide R.S. (Carlo Erba, Milan, Italy), a 1 *M* buffer solution (pH 9) of boric acid–sodium carbon-

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ate–potassium chloride and a 1 *M* phosphate buffer solution (pH 5) and ketodase enzyme (Warner-Chilcott, Morris Plain, N.J., U.S.A.). All of the reagents were of analytical reagent grade (purity 99%) and all of the inorganic reagents were made up in doubly distilled water. Acetonitrile (Uvasol; E. Merck, Darmstadt, G.F.R.) and doubly distilled water used as eluents in liquid chromatography were prefiltered by use of a 2-μm filter.

Apparatus

A Hewlett-Packard Model 1084 A liquid chromatograph equipped with a built-in fixed-wavelength (254 nm) high-sensitivity UV detector was used.

The Hewlett-Packard column (stainless steel, 250 mm) was packed with LiChrosorb RP-8 (particle size, 10 μm). The chromatographic conditions were as follows: mobile phase, water–acetonitrile (45:55); flow-rate, 0.8 ml/min; pressure, 38 atm; column temperature, 40°.

The extraction procedure for serum and urine samples was as described previously[1].

HPLC analysis

The residue from the extraction after evaporation of the solvent was dissolved in 200 μl of acetonitrile. 300 ng of diazepam (dissolved in acetonitrile) were added to the resulting solution as the internal standard. 20 μl of this solution were injected into the liquid chromatograph. The calibration curves (Fig. 2) were obtained by plotting the ratio of the peak areas against the amounts of solution injected into the liquid chromatograph. The peak areas were calculated by multiplying the peak heights by the peak widths at half-height.

The four metabolites of pinazepam and the internal standard were completely separated as can be seen from the chromatograph of a standard mixture of these five

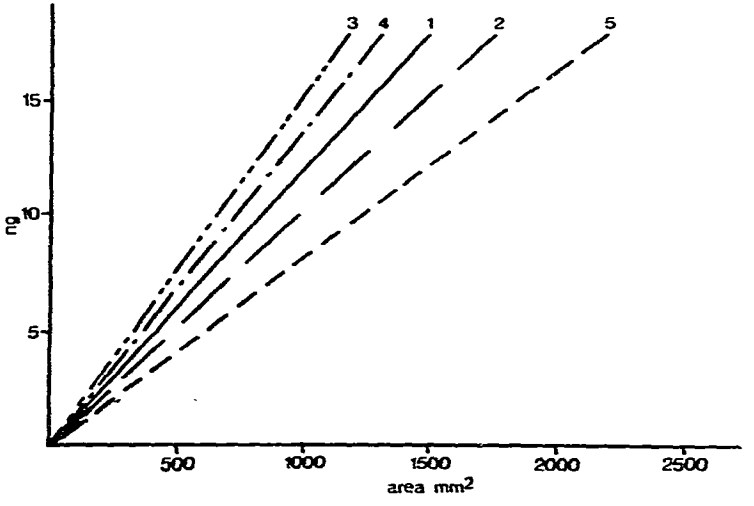

Fig. 2. Calibration curves for oxazepam (1), desmethyldiazepam (2), 3-hydroxypinazepam (3), diazepam (4) and pinazepam (5).

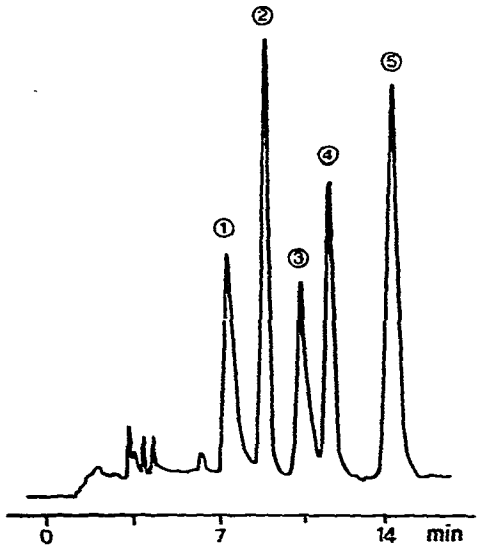

Fig. 3. Chromatogram of the standard mixture of the five substances. Peaks as in Fig. 2.

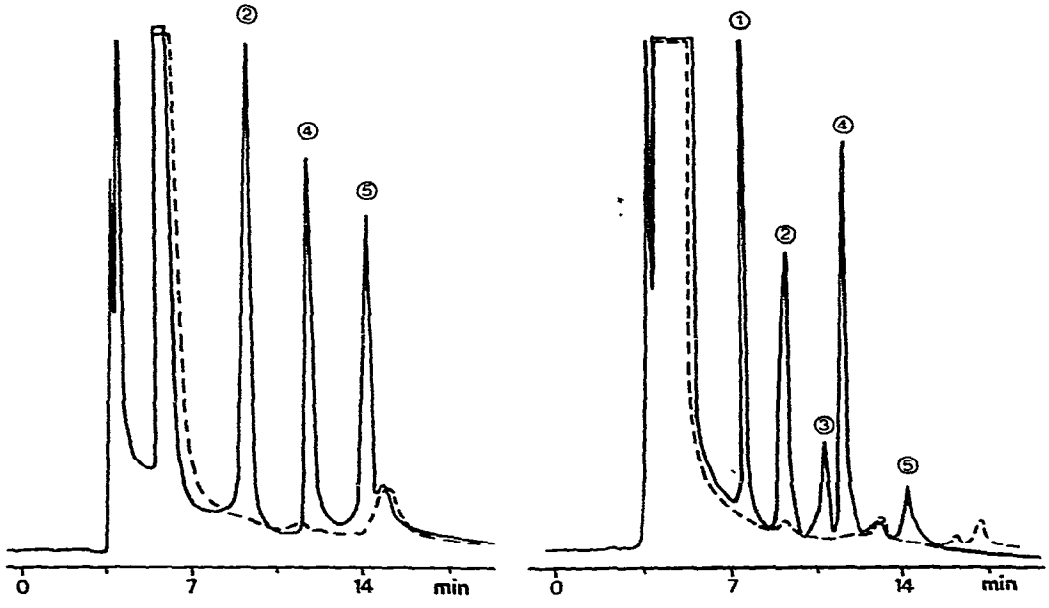

Fig. 4. Chromatogram of human serum extract. Peaks as in Fig. 2. Broken line, blank extract.

Fig. 5. Chromatogram of human urine extract. Peaks as in Fig. 2. Broken line, blank extract.

compounds (Fig. 3). Chromatograms of human serum and urine extracts are compared with those of blank extracts in Figs. 4 and 5, respectively. The peaks of pinazepan and its metabolites in the samples were identified by comparison of their retention times and those of the standard mixture. The retention times are listed in Table I.

TABLE I

RETENTION TIMES

Substance	t_R *(min)*
Pinazepam	14.60
Desmethyldiazepam	8.70
Oxazepam	7.00
3-Hydroxypinazepam	10.35
Diazepam	11.68

Recovery and sensitivity

The recovery was 84% for pinazepam, 79% for desmethyldiazepam, 83% for oxazepam and 76% for 3-hydroxypinazepam. Since the minimum detectable amount of each metabolite was *ca.* 2 ng for a single determination, drug levels as low as 20 ng/ml of serum can be detected.

It can be concluded that, for the purposes of the present investigation, HPLC is as sensitive as gas chromatography. However, determinations made by HPLC are simpler and less time-consuming than those made by gas chromatography.

REFERENCES

1 A. Trebbi, G. B. Gervasi and V. Comi, *J. Chromatogr.*, 110 (1975) 309; see also G. M. Pacifici and G. F. Placidi, *J. Chromatogr.*, 135 (1977) 133.