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Note

A simplified quantitative method for the simultaneous determination of diazepam and its metabolites in serum by thin-layer chromatography

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Diazepam and its metabolites in serum are usually measured quantitatively by gas chromatography (GC) using electron-capture[1] or flame-ionization[2] detection. As an alternative to GC, we have developed a simple and sensitive thin-layer chromatographic (TLC) method. We measure directly from an unstained thin-layer plate the diffuse-light reflectance of diazepam and its metabolites at their absorption maximum.

MATERIALS AND METHODS

Apparatus

A BTL shaker (Baird & Tatlock Ltd., London, Great Britain), a compressed air evaporation system, a Hamilton precision syringe (50 μl), a thin-layer chromatographic tank, screw-capped tubes (130 × 13 mm, Sovirel) and a Vortex-Genie mixer were used. A chromatogram-spectrophotometer KM 3 (Carl Zeiss, Oberkochen, G.F.R.) was set up for light reflection measurement as follows: hydrogen lamp–monochromator–sample–detector.

Materials

The pre-coated silica gel 60 TLC plates (without a fluorescent indicator) had dimensions of 20 × 20 cm and a layer thickness of 0.25 mm (Merck).

The following reagent-grade chemicals and solvents were used: toluene, diethyl ether, chloroform, *n*-heptane, absolute ethanol and glycine. All drugs were kindly supplied by Hoffmann-La Roche & Co., Basle, Switzerland.

Stock standard solution (25 mg per 100 ml). Amounts of 25 mg each of diazepam (RO 5-2807), N-desmethyldiazepam (RO 5-2180), 3-hydroxydiazepam (RO 5-5345) and oxazepam (RO 5-6789) are dissolved in 100 ml of absolute ethanol. This solution is stable for at least 6 months at room temperature.

Working standard solution (2.5 mg per 100 ml). This solution is prepared by 1:10 dilution of the stock standard solution with absolute ethanol.

Procedure

Serum (1 ml) is placed in a 130 × 13 mm Sovirel screw-capped tube, 0.5 ml of 2 *M* glycine buffer (pH 10.5) and 8 ml of toluene are added. The tube is

tightly closed and shaken vigorously for 5 min with a BTL shaker. The tube is then centrifuged for 20 min at 1400 *g*. A 7-ml volume of the toluene phase is transferred into a conical centrifuge tube, placed in a 60° water-bath and evaporated to dryness by means of a direct air stream. The residue is cooled to room temperature and dissolved in 50 μl of chloroform.

The entire solution obtained is applied to a thin-layer plate with a Hamilton syringe to a width of 0.8 cm and 1.5 cm from the bottom. It was found that eight samples could be applied on a 20 × 20 cm thin-layer plate. The thin-layer plate is then placed in an unlined glass chromatography tank containing 100 ml of chloroform–diethyl ether (60:40) and is allowed to develop for a distance of 14 cm at room temperature. This procedure transfers interfering natural lipophilic compounds in the serum to the solution front. The dried plate is then developed at room temperature in chloroform–*n*-heptane–absolute ethanol (50:50:5)[3]. A total of 2.5 h is needed for the two development steps.

A standard graph is prepared by adding 5, 10, 20 and 40 μl each of the working standard solution to 1 ml of serum free of drugs and extracting with the above procedure. The highest point in this curve corresponds to a concentration of 1 μg/ml in serum, as shown in Fig. 1.

The thin-layer plate is scanned at the wavelength of 230 nm for diffuse reflectance with the Zeiss chromatogram-spectrophotometer.

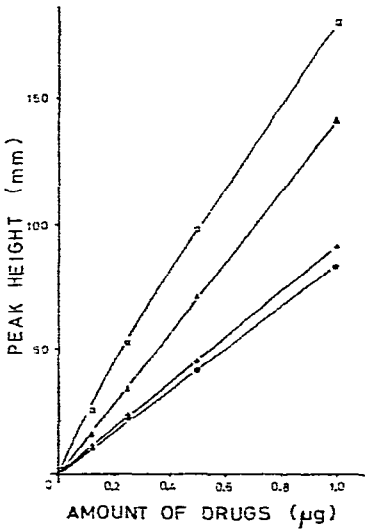

Fig. 1. Standard graphs for diazepam (+), N-desmethyldiazepam (□), 3-hydroxydiazepam (●) and oxazepam (▲) extracted from serum.

RESULTS AND DISCUSSION

One of the advantages of this TLC method compared with GC methods is the simpler extraction of the native medicament and its metabolites from serum[1,2].

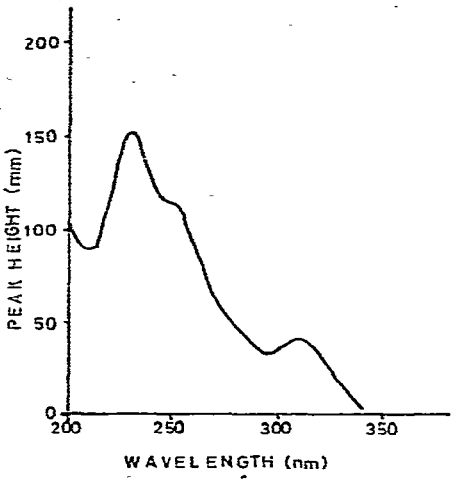

Fig. 2. Ultraviolet absorption spectrum of a thin-layer chromatogram containing diazepam, N-desmethyldiazepam, 3-hydroxydiazepam or oxazepam, applied directly to the plate, separated chromatographically and measured *in situ* with the Zeiss chromatogram-spectrophotometer.

Only one extraction is necessary in the TLC method and the extracted substances are chromatographed without the need for derivative formation. A major advantage

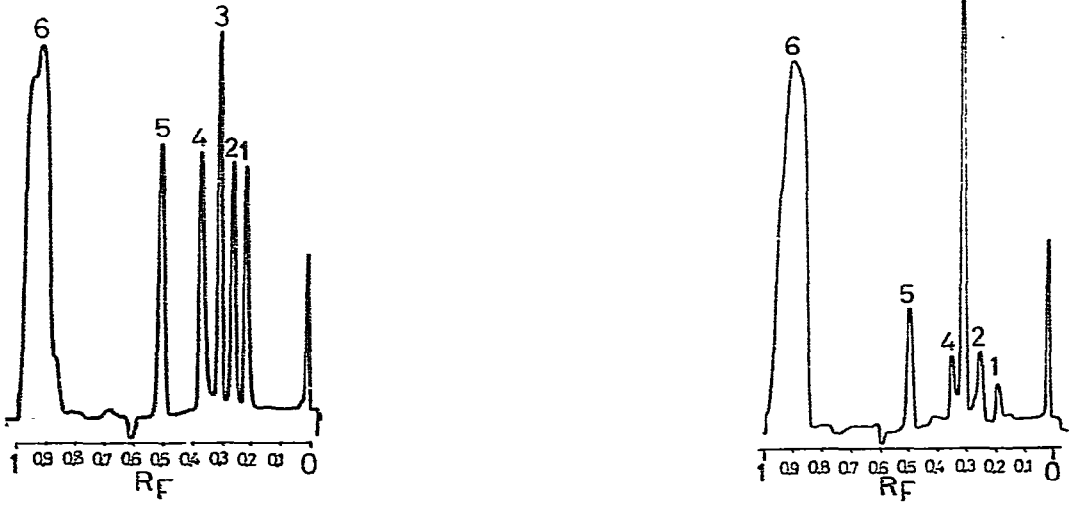

Fig. 3. Results obtained after a scan at 230 nm of the TLC separation of 1 ml of serum to which had been added 0.5 μg/ml each of diazepam and its metabolites. Peaks: 1, oxazepam; 2, caffeine; 3, N-desmethyldiazepam; 4, 3-hydroxydiazepam; 5, diazepam; 6, the solution front containing natural lipophilic compounds.

Fig. 4. Results obtained after a scan at 230 nm of the TLC separation of 1 ml of serum from a patient. Peaks: 1, oxazepam; 2, caffeine; 3, N-desmethyldiazepam; 4, 3-hydroxydiazepam; 5, diazepam; 6, the solution front containing natural lipophilic compounds.

TABLE I

RECOVERY OF DRUGS FROM SERUM AND REPRODUCIBILITY OF THE METHOD

Drug	*Recovery (%)*	*Reproducibility (30 samples)*	
		Mean ± s.d. (μg/ml)	*Coefficient of variation (%)*
Diazepam	92.7	0.80 ± 0.04	5.4
N-Desmethyldiazepam	93.9	0.47 ± 0.02	4.7
3-Hydroxydiazepam	99.0	0.42 ± 0.01	2.4
Oxazepam	86.8	0.51 ± 0.04	8.5

of the TLC method over GC methods is the possibility of directly scanning the spots on the thin-layer plate over the UV range to obtain the absorption spectrum and then comparing this pattern with known absorption spectra for positive identification. Diazepam and its metabolites exhibit the absorption spectrum shown in Fig. 2.

The utilization of time in the TLC method was found to be more efficient than in GC methods. There are approximately 2.5 h of free time during the two developments of an eight-sample thin-layer plate, compared with 22 min in one GC separation of diazepam and its metabolites[1].

The chromatographic separation of the working standard solution added to 1 ml of serum is shown in Fig. 3. The chromatographic separations of serum extracts from most of our patients (Fig. 4) exhibited a peak at R_F 0.25, which was found to be caffeine.

A survey of 30 serum samples demonstrates the recovery and the reproducibility of the method (Table I).

We conclude that this quantitative TLC method is rapid and precise and the results are well within the accepted limits of deviation.

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