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Note

Simplified thin-layer chromatographic method for the simultaneous determination of clonazepam, diazepam and their metabolites in serum

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When epileptic patients are admitted as emergency cases, they are usually given diazepam and, if the desired response is not observed, then clonazepam is often administered. This clinical procedure necessitates a method for the simultaneous and quantitative measurement of the two drugs and their metabolites.

Clonazepam [1], diazepam [2] and their metabolites are usually quantitatively measured separately by gas chromatography using electron-capture detection. As an alternative to this lengthy procedure, we have applied the method of Wad et al. [3], in which the diffuse light reflectance of both substances and their metabolites which have been simultaneously extracted and separated are measured directly on an unstained thin-layer chromatographic (TLC) plate.

METHOD

The extraction and separation were carried out according to the method of Wad et al. [3]. We are listing some precautions that are necessary for the optimal separation of the eight drugs when using this method.

The drugs are applied to the plate at a width of 0.8 cm and to a height of 2—3 mm. An application of height less than 2 mm will result in incomplete separation of the two metabolites of clonazepam.

The first separation solvent, chloroform—diethyl ether (60:40), is used primarily for the removal of natural interfering substances, but it also initiates the separation of the drugs. Thus development of this first separation phase by more than 14 cm will result in the merging of oxazepam with caffeine

and of N-desmethyldiazepam with 3-hydroxydiazepam.

The final solvent, chloroform—*n*-heptane—ethanol (50:50:5), must be developed to the top of the plate; if not, incomplete separation of oxazepam and 7-aminoclonazepam will occur.

To the stock standard solution (25 mg per 100 ml) were added 25 mg each of clonazepam, 7-aminoclonazepam and 7-acetamidoclonazepam (donated by F. Hoffmann-La Roche, Basle, Switzerland). This solution was then diluted 1:10 with absolute ethanol to give a 2.5 mg per 100 ml working standard solution. The standard curves for these additional drugs are shown in Fig. 1.

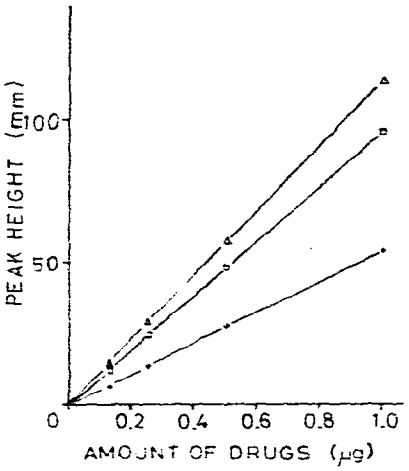

Fig. 1. Standard curves for clonazepam (△), 7-aminoclonazepam (□) and 7-acetamidoclonazepam (+) extracted from serum, expressed as peak height versus amount.

The scanning for diffuse light reflectance was executed at 230 nm for diazepam and its metabolites and at 250 nm for clonazepam and its metabolites by means of a Zeiss chromatogram-spectrophotometer.

RESULTS AND DISCUSSION

One of the advantages of our TLC method compared with GC methods is the ease of extraction of the native medicaments and their metabolites from serum [1, 2]. Only one extraction is necessary and the extracted substances are chromatographed without any derivatization. A major advantage over GC methods is the possibility of scanning the spots on the TLC plate directly in the UV range in order to obtain the absorption spectrum and then comparing this pattern with known absorption spectra for positive identification. Diazepam and its metabolites exhibit the same absorption spectrum whereas clonazepam and its metabolites exhibit dissimilar absorption spectra. Absorption spectra of clonazepam, diazepam and their metabolites are shown in Fig. 2.

Time is employed more efficiently in our TLC method than in GC methods: there is approximately 2.5 h of free time during the two developments of

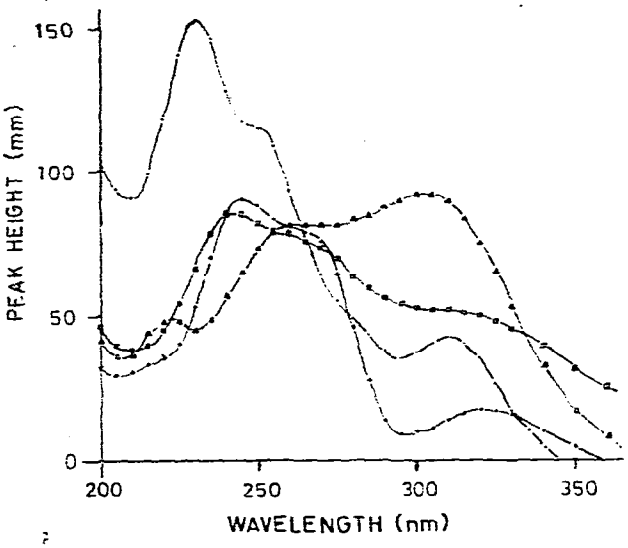

Fig. 2. Ultraviolet absorption spectra obtained by scanning a TLC plate containing 0.5 μg each of clonazepam (△), 7-aminoclonazepam (□), 7-acetamidoclonazepam (+), diazepam and the three metabolites of diazepam (●) which were applied directly on to a plate, chromatographically separated and measured in situ by means of a Zeiss chromatogram-spectrophotometer.

an eight-sample TLC plate, compared with 22 min for one GC separation of diazepam and its metabolites [2]. Clonazepam and its metabolites must be measured separately with GC [1]. Clonazepam is hydrolyzed into its corresponding benzophenone and then measured by GC, whereas the metabolites of clonazepam are measured directly without hydrolysis. The total time for these two GC runs is approximately 12 min.

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

We have analyzed sera from 39 patients for therapeutic control of clonazepam using our TLC method. Only 12 of these sera exhibited a calculable peak for all three serum constituents of clonazepam. The mean values for these 12 sera were 62 ng/ml for clonazepam, 243 ng/ml for 7-aminodiazepam and 420 ng/ml for 7-acetamidodiazepam.

Interference from other anticonvulsive drugs administered in our hospital has not been observed. We have found only carbamazepine and sulfamethoxazol (a component of Bactrim) to be extracted and separated by this method, but they create no problems as they both have the same R_F value as caffeine.

The recovery and reproducibility of the method are presented in Table I. The recoveries were obtained by comparing the drugs directly applied in the same amounts as the drugs being extracted from serum, applied and separated. The reproducibility is the result of 30 analyses of the same serum sample to which the five drugs were added.

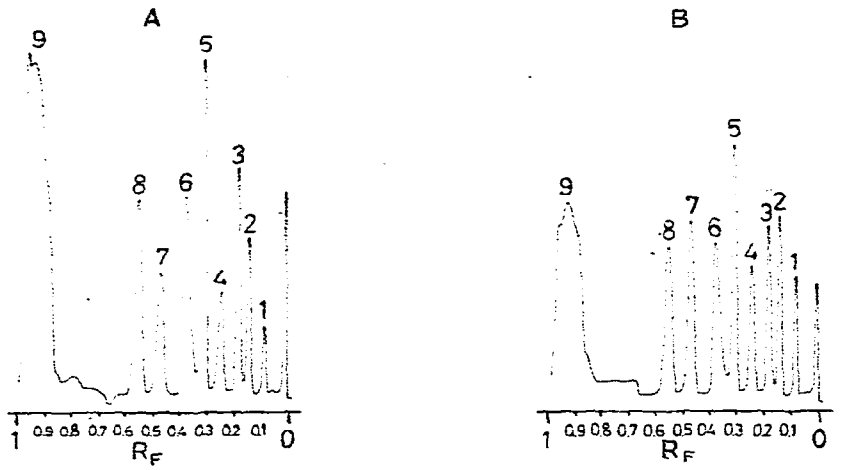

Fig. 3. Results obtained from scans at 230 nm (A) and 250 nm (B) of a TLC plate after the separation of 1 ml of a drug-free serum to which had been added 0.5 μg/ml each of diazepam, clonazepam, and their metabolites. The solvent system was chloroform—diethyl ether (60:40) followed by a second separation in chloroform—*n*-heptane—ethanol (50:50:5) [3]. Peaks: 1 = 7-acetamidoclonazepam; 2 = 7-aminoclonazepam; 3 = oxazepam; 4 = caffeine; 5 = N-desmethyldiazepam; 6 = 3-hydroxydiazepam; 7 = clonazepam; 8 = diazepam; 9 = solution front containing the naturally occurring substances in serum.

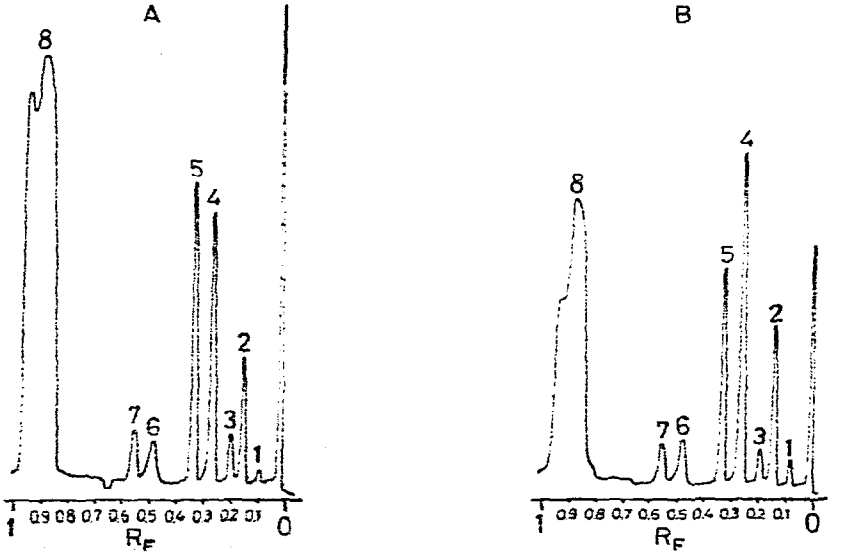

Fig. 4. Results obtained from scans at 230 nm (A) and 250 nm (B) of a TLC plate after the separation of 1 ml of serum of a patient. The solvent system was chloroform—diethyl ether (60:40) followed by a second separation in chloroform—*n*-heptane—ethanol (50:50:5) [3]. Peaks: 1 = 7-acetamidoclonazepam; 2 = 7-aminoclonazepam; 3 = oxazepam; 4 = caffeine; 5 = N-desmethyldiazepam; 6 = clonazepam; 7 = diazepam; 8 = solution front containing the naturally occurring substances in serum.

TABLE I

RECOVERY OF THE DRUGS FROM SERUM AND REPRODUCIBILITY OF THE METHOD

Drug	Recovery (%)	Reproducibility (30 samples)	
		Mean ± S.D. (μg/ml)	C.V. (%)
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
Clonazepam	98.6	0.56 ± 0.03	5.2
7-Aminoclonazepam	77.1	0.59 ± 0.04	6.8
7-Acetamidoclonazepam	40.3	0.51 ± 0.03	4.8

It can be concluded that our quantitative TLC method is rapid and precise and the results are well within the accepted limits of deviation.

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3 N. Wad, H. Rosenmund and E. Hanifl, J. Chromatogr., 128 (1976) 231.