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Quantitative determination of diazepam, nitrazepam and flunitrazepam in tablets using thin-layer chromatography–densitometry technique

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Abstract

A simple and reliable assay for diazepam, nitrazepam and flunitrazepam in tablets by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC)–densitometry is described. A quantity of a ground tablet mass, equal to the average weight of one tablet was sonicated in MeOH, mixed with appropriate internal standard, filtered and either injected directly into the liquid chromatograph, or after evaporation and reconstitution of an aliquot of the extract, was spotted on a silica gel thin-layer plate. A variable UV detector, operated at 254 nm was employed in both procedures. A C18, reversed phase 7 μm column was used for HPLC analysis; the mobile phase was a 1:1 (v/v) mixture of MeOH (40 °C) and 0.01 M phosphate buffer (pH 7, 80 °C). The TLC plate was developed in an unsaturated chromatographic chamber containing 100 ml chloroform–acetone (9:1); at room temperature, the mobile phase was allowed to travel 15 cm. The percentage of the active ingredient content of each tablet obtained by both procedures, was in the range of the stated amount except for one brand of diazepam tablets which contained ~23% less active ingredient than the minimum prescribed amount. The TLC densitometry, although yields slightly higher values than the HPLC method, is preferred due to its simplicity, ease and low cost.

Keywords: Benzodiazepines; Reversed phase HPLC; TLC–densitometry

1. Introduction

The benzodiazepines are among the most frequently prescribed drugs for the treatment of sleep disturbance and anxiety [1]. They act on the central nervous system and exhibit hypnotic tranquilizing and anticonvulsant properties [2–5].

Used singly, these drugs appear to be relatively safe, but are of significant pharmacological importance when combined with other depressant drugs such as barbiturates, narcotics and phenothinzines [6]. The widespread use of these drugs

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necessitates a rapid and reliable method for their determination.

Several analytical methods are available for determining benzodiazepines and their metabolites in body fluids [7–17]. These methods, however, have rarely been applied for quantitation of the intact benzodiazepines in pharmaceutical formulations. Instead, the pharmacopeial procedures are the currently common methods of analysis; although such procedures have already been criticized as being tedious, time consuming with occasionally lack of specificity [18–21]. Spectrophotometry [22], electrochemical methods [23], high performance liquid chromatography [24–26], capillary electrophoresis [27] gas chromatography [28] and thin layer chromatography (TLC)–densitometry [29–31] are current methods for the quantitation of some of the benzodiazepines in pharmaceutical formulations.

Of these techniques, liquid chromatography, offers more possibility for adjusting selectivity [32,33]. Furthermore, because of the low working temperature, it appears to be particularly suitable for the analysis of thermally labile compounds. However, time consuming pre-treatment of sample is mandatory before injection into the HPLC columns. Alternatively, quantitative TLC with in situ scanning densitometry is rapidly gaining wide acceptance in pharmaceutical analysts [34–36]. This is because of its simplicity, accuracy, cost-effectiveness and the possibility of simultaneous determination of a number of samples on a single TLC plate.

This paper describes a simple, rapid methodology for the quantitation in pharmaceutical formulations of diazepam, nitrazepam and flunitrazepam drugs of forensic interest using TLC–densitometry. The objectives of this study was to develop quantitative procedures for the determination of all three drugs, in order to differentiate between the legally prescribed and their illicitly produced formulations in solid dosage form by comparing their active ingredient content. The results are compared with those obtained using HPLC.

2. Experimental

2.1. Materials

The benzodiazepines were obtained from Hoffmann–La Roche, Basle, Switzerland. Commercially coated silica gel Merck plates were used: 20×20 cm, silica gel 0.2 mm in thickness. Methanol, distilled and deionised water were further purified by passing through the appropriate purification system (Millipore, Bedford, MA, USA) before use.

2.2. HPLC analysis

2.2.1. Instrumentation

A Hewlett–Packard Model 1084B liquid chromatograph equipped with an electronic integrator, a dual wavelength UV detector and a Hibar column (250×4 mm I.D.) prepacked with Li-Chrosorb RP-18 (mean particle size 7 mm) from Merck was used for HPLC analysis.

2.2.2. Chromatographic conditions

The mobile phase was a 1:1 (v/v) mixture of methanol (40 °C and 0.01 M phosphate buffer (pH 7, 80 °C). Other conditions were as follows: flow rate 2 ml min^{-1}, oven-temperature 60 °C, injection volume 10 μl and detection wavelength 254 nm.

2.2.3. Standard for calibration graphs

Stock solutions of diazepam, nitrazepam and flunitrazepam (1 mg ml^{-1}) were prepared by dissolving of the appropriate amounts in methanol. Diazepam was used as an internal standard for both nitrazepam and flunitrazepam, whereas nitrazepam was the internal standard for diazepam.

Working standards were prepared by dilution of the stock solution (1.25, 2.5, 5, 10 and 20 ml) with methanol and 5 ml of the appropriate internal standard solution to a final volume of 50 ml. These correspond to working standards of 25, 50, 100, 200 and 400 μg ml^{-1}. Calibration plots of the standards prior to analysis to establish the linearity and reproducibility of the HPLC system were constructed by plotting the average peak–height

ratio of each compound to internal standard against drug concentration. The average peak–height ratios were mean of two runs for each standard solution.

2.2.4. Unknown preparations

Five tablets each of the various dosage forms of 2, 5 and 10 mg of diazepam, 5 mg nitrazepam and 2 mg flunitrazepam were obtained from different sources were weighed and then ground to a fine powder. In each case a quantity of the powder equal to the average weight of one tablet was mixed with 5 ml of the appropriate internal standard solution and diluted to a final volume of 50 ml with methanol and sonicated for 5 min.

2.3. Scanning densitometry

2.3.1. Apparatus

A Shimadzu high speed TLC scanner CS-920 equipped with an electronic integrator, a TLC spotting device with 0.75 μl precision capillary tubes from Merck, screw capped vials (Pierce Chemical Corp., Illinois, USA) and a thermolyne mixer were used for this experiment.

2.3.2. Standards

In this study nitrazepam was used as an internal standard for both diazepam and flunitrazepam whereas diazepam was the internal standard for nitrazepam. Volumes of 2 and 4 ml of the stock solution were mixed with 3 ml of the corresponding internal standard. The mixture was then evaporated under a stream of nitrogen and the residue was reconstituted with 5 μl of purified methanol.

2.3.3. Unknown preparation

Five microliter of each tablet extract that was used for HPLC was quantitatively transferred to a respective vial. Aliquots of 0.3, 0.6 and 1.2 ml of the standard solution were added subsequently to the extracts of 2, 5 and 10 mg tablets. The mixture was then evaporated under nitrogen and the residue was reconstituted with 5, 10 and 20 μl methanol, respectively.

2.3.4. Chromatographic procedures

Standard and unknown solution (0.75 μl each) was applied onto a preactivated thin layer plate (at 120 °C for 10 min) with the spotting device to a width of 1.5 cm from each other and 3 cm from the bottom of the plate. After drying the spots in a stream of nitrogen, the TLC plate was developed in an unsaturated TLC tank containing 100 ml of chloroform–acetone (1:1); this mobile phase was allowed to travel 15 cm. After evaporation of solvents, the spots of each lane were scanned at 254 nm using the two-point calibration mode. These readings were reproducible over consecutive runs.

3. Results and discussion

Considering the instability of the benzodiazepines towards acidic or alkaline conditions at elevated temperature, the pH of the mobile phase for HPLC analysis was buffered to 7.01. At this pH, other variables, which influence a HPLC analysis, were monitored systematically. The optimal chromatographic conditions adopted were stated in the Section 2. Calibration studies showed linearity of response as measured by relative peak height in the range of concentrations used in the experiment. Reproducible values were obtained over repeated runs yielding good calibration curves passing through the origin.

The response of the TLC-densitometer to each benzodiazepine in on-plate quantitative analysis was determined at 5 nm interval from 230 to 350 nm. The optimal wavelength for the quantitation of the benzodiazepines was chosen to be 254 nm. At this wavelength, varying concentrations of the benzodiazepines on the same plate yields, reproducible data with respect to relative peak area measurements.

The results of the quantitative analysis of the benzodiazepines in different tablets with regards to both chromatographic techniques are tabulated in Table Table 1. The results obtained indicate that the active ingredient content of each tablet ob-

Table 1
Results for the determination of active ingredients of some benzodiazepine tablets using the HPLC and TLC densitometry method

Manufacturer	Active Ingredient And Dosage	Recoveries HPLC	TLC - Densitometry
A	Diazepam (2 mg)	101.0	108.0
B	Diazepam (2 mg)	98.0	105.6
C	Diazepam (2 mg)	98.0	108.3
A	Diazepam (5 mg)	97.5	102.7
C	Diazepam (5 mg)	103.4	106.0
D	Diazepam (5 mg)	69.0	72.0
B	Diazepam (10 mg)	92.7	98.3
C	Diazepam (10mg)	69.5	73.7
A	Nitrazepam (5 mg)	87.9	94.2
A	Flunitrazepam (2 mg)	105.1	107.5

* $n \geq 2$

tained by on-plate quantitative TLC analysis is slightly higher compared with the values obtained by HPLC analysis. This difference can well be explained on the basis of applying two different procedures with different sensitivities and also by considering the fact that in scanning densitomeiry, two-point calibration is used for quantitative measurements while in HPLC, multiple point calibration measurement curve is used for this purpose. A plot of the recoveries between the two methods is shown in Fig. 1. A correlation coefficient of 0.9697 was obtained. However, the percentage of the active ingredient content of each tablet is in the range of the standard amount, except for one type of diazepam tablets, although obtained through a legitimate source, but contains much less active ingredients than the prescribed amount.

Both procedures permit a fast and reliable determination of these drugs in pharmaceutical dosage forms and can be used for routine analysis. However, the scanning densitometry is superior in terms of speed, simplicity and cost.

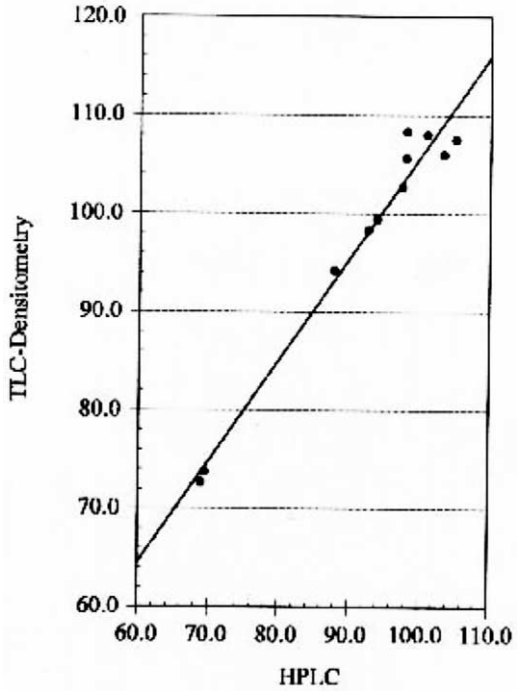

Fig. 1. Correlation in the recovery for the determination of some benzodiazepine tablets as determined using HPLC and TLC-densitometry

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