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DENSITOMETRIC EVALUATION OF DIAZEPAM IN PHARMACEUTICAL PREPARATIONS

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SUMMARY

Eleven densitometric methods of quantitative determination of diazepam have been compared, using oxazepam as internal standard in seven of them; in this way interference of degradation products may be eliminated. The work has been conducted in a rather simple way: an inexpensive densitometer which gave transmission values was used, only five replicates being made for the statistical calculation. In all cases good correlation coefficients between peak areas and concentration in the range of 10 to 50 μg by spot were found. The accuracy was very good in all methods; the precision (s_r) was from 1 to 3% in some methods. The results could be greatly improved with better instrumentation and more replicates.

INTRODUCTION

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one) and its metabolites have been studied more intensively than the other benzodiazepines. The official compendia BP[1], NF XIII[2], USP XIX[3], etc., propose spectrophotometric methods for the evaluation of diazepam in pharmaceutical preparations. Baggi *et al.*[4] reported a colorimetric method for estimation of diazepam in pharmaceutical preparations, without taking into account the degradation products. Beyer and Sadee[5] titrated different benzodiazepines in non-aqueous media with 0.01 *N* perchloric acid; the NF XIII describes the evaluation of diazepam by this method. In tests of accelerated aging we have found 2-methylamino-5-chlorobenzophenone (MACB) as a degradation product of diazepam.

For studies of the expiration date of diazepam in pharmaceutical preparations, it is necessary to use a method for the quantitative analysis of diazepam which in turn could eliminate the interference of any of the degradation products. Thin-layer chromatography (TLC) and the evaluation via densitometric methods have been chosen for simplicity and efficiency. This paper describes the comparison of different densitometric methods found in the literature (Table I), determining both the precision (relative standard deviation), $s_r = s/\bar{x} \cdot 100$, and accuracy (relative error), $[(\bar{x}-\bar{x}_r)/\bar{x}_r] \cdot 100$, where $\bar{x}_r$ is the actual value.

TABLE I

DENSITOMETRIC METHODS IN THE LITERATURE

Method		*Reference*
A	Internal or direct calibration	9; "direkte Analyse, direkte Auswertung" 12; "individual calibration, direct thin-layer evaluation"
A1	No internal standard	
A1a	Simple method	9; "Eichfunktion"
A1b	Data pair technique	11; "data pair technique"
A2	Internal standard	13; "interner Standard", "internal standard"
A2a	With calibration curve	14; "Eichkurve"
A2b	With calibration factor	10; "Eichfaktor"
B	External or transferable calibration	14; "übertragbare Eichkurven" 12; "external calibration, transferable calibration, transferable calibration curves"
B1	No internal standard	
B1a	Simple method	
B1b	Transfer of mean slope	12; "direct transfer factor"
B1c	Transfer of relative slope	12; "transfer of relative slope"
B2	Internal standard	
B2a	Simple method	15; "méthode de l'étalon interne"
B2c	Transfer of relative slope	16
B2d	Transferable calibration factor	16

MATERIALS AND METHODS

Materials

Diazepam and oxazepam were products from Impex Quimica (Barcelona, Spain); Merck p.a. solvents were used.

Thin-layer chromatography

Ascending TLC was carried out on silica gel G (Merck) plates (20 × 20 cm, layer thickness 0.25 mm), coated with a Uniplan spreader (Shandon, London, Great Britain). Chromatographic tanks (Shandon, SAB 2849) lined with filter-paper were used. The saturation time was 15 min. The plates were dried at room temperature overnight. The solvent system used was *n*-heptane–chloroform–absolute ethanol (10:10:1)[6–8]. Development was for 16.5 cm (60 min). The R_F values corresponding to diazepam, MACB and oxazepam were 0.33, 0.72 and 0.11, respectively.

Concentrated hydrochloric acid–absolute ethanol (1:1) was used as spray reagent. On heating the plate in an oven at 100° for 30 min, diazepam and oxazepam appeared as yellow and orange spots, respectively. The densitometric values were constant for 1 h; they were read after 30 min in transverse direction. The measurements were carried out with a Model 509 Photovolt densitometer, with automatic recording. Conditions: transmitted light; attenuation × 3; speed of paper 10 mm/min. The reading of the region next to the spot was taken as blank. The densitometric peak areas were measured with a planimeter (Rost, Wien, Austria), and the mean of two measures of each peak was used.

Densitometric methods

The different densitometric methods found in the literature are given in Table I.

(A) Internal or direct calibration. A calibration curve was made from one plate for the evaluation of one or several spots chromatographed on the same plate. It is the most suitable method for a few analyses. Five replicates were made for the determination of precision and accuracy.

(1) No internal standard. In the simple method (a), a calibration curve is prepared from the values obtained with one plate, plotting the relationship between area and concentration. The concentration of an unknown spot is determined by interpolation.

In the data pair technique (b), a series of standard concentrations is applied on a plate. The procedure is repeated on the same plate with the same series of standards but with an equal amount of the unknown. The concentration of the unknown is then calculated from the intercepts of the curves with the concentration axis.

(2) Internal standard. With the calibration curve method (a), to each standard concentration an equal amount of internal standard is added on the same plate. The ratio of the areas

$$R = \frac{\text{area of standard}}{\text{area of internal standard}} \tag{1}$$

is plotted against concentration. The unknown is treated in the same way.

In the calibration factor method (b), the factors f and f' are determined from known amounts of internal standard (c_{is}) and standard (c_s)

$$f = \frac{c_s}{c_{is}} \cdot \frac{A_{is}}{A_s}; \qquad f' = f \cdot c_{is} = c_s \cdot \frac{A_{is}}{A_s} \tag{2}$$

where A_{is} = area of internal standard and A_s = area of standard. An equal amount of internal standard is then added to the unknown and the concentration of the latter determined from

$$c = f' \cdot \frac{A}{A'} \tag{3}$$

where A = area of the unknown and A' = area of internal standard, added to the unknown.

(B) Transferable or external calibration. A transferable calibration curve is made from a series of plates in order to determine one or several spots, applied to other plates. This is the most suitable method for a long series of analyses. Five plates were made for obtaining the calibration curve and five more for determining the precision and accuracy.

(1) No internal standard. In the simple method (a), the mean area of the peaks corresponding to each concentration is plotted against concentration. The concentration of the unknown is determined by interpolation.

In the transfer of mean slope (b), calibration curves are prepared and the mean slope ($\bar{b}$) for all curves is determined.

A known amount of standard is applied on another plate, giving an area A. The ordinate intercept (a) is determined:

$$A = a + \bar{b} \cdot c \tag{4}$$

With these values of a and $\bar{b}$ the concentration of the unknown may be obtained. The unknown is treated in the same way. Five plates were made for determining the mean slope ($\bar{b}$), one more for calculating the ordinate intercept (a) and five more for finding the precision and accuracy.

In the transfer of relative slope (c), the respective calibrations curves are prepared from values obtained on several plates. The relative slope (b_{rel}) for each plate is determined:

$$b_{rel} = \frac{b_i}{A_i/n}; \qquad A_i = A_1 + A_2 \ldots\ldots\ldots + A_n \tag{5}$$

where A_i = sum of areas of standard corresponding to each concentration, b_i = slope of each curve and n = number of concentrations. The mean slope of all plates ($\overline{b_{rel}}$) is also calculated. A known amount of standard (c) is applied on another plate, giving an area A. The ordinate intercept (a) is determined:

$$A = a + b \cdot c; \text{ where } b = A \cdot \overline{b_{rel}} \tag{6}$$

With these values of a and b the concentration of the unknown may be obtained. Five plates were made for determining the mean relative slope ($\overline{b_{rel}}$), one for calculating the ordinate intercept (a) and five more for finding the precision and accuracy.

(2) Internal standard. In the simple method (a), an equal amount of internal standard is added to each standard concentration on all plates. For each plate and for each concentration, the ratio of areas, R, is calculated as in eqn. 1, and the mean ratio of areas, $\bar{R}$, is determined and plotted against concentration. The unknown is treated in the same way.

In the transfer of mean slope (b), an equal amount of internal standard is added to each standard concentration on each plate. The calibration curves are prepared from values obtained on several plates and the mean slope ($\bar{b}$) for all curves is determined. A known concentration of standard (c) with the same amount of internal standard is applied on another plate, which will give a ratio of areas (R). The ordinate intercept (a) is determined:

$$R = a + \bar{b} \cdot c$$

With these values of a and $\bar{b}$ the concentration of the unknowns may be obtained. Five plates were made for determining the mean slope ($\bar{b}$), one for calculating the ordinate intercept (a) and five more for finding the precision and accuracy.

In the transfer of relative slope (c), .n equal amount of internal standard is

added to each standard concentration on each plate. The calibration curves are made from values obtained on several plates. The ratio of areas, R, is plotted against concentration. For each plate, the relative slope ($\overline{b_{rel}}$) is determined:

$$b_{rel} = \frac{b_i}{R_i/n}; \qquad R_i = R_1 + R_2 \ldots\ldots\ldots + R_n \tag{7}$$

where R_i = sum of relationship of areas, corresponding to each standard concentration, b_i = slope of each curve and n = number of concentrations. The mean slope of all plates ($\overline{b_{rel}}$) is also calculated. A known concentration of standard (c) with the same amount of internal standard is applied on another plate, which will give a ratio of areas (R). The ordinate intercept (a) is determined:

$$R = a + b \cdot c; \quad b = \overline{b_{rel}} \cdot R \tag{8}$$

With these values of a and b the concentration of the unknowns may be obtained. Five plates were made for determining the mean relative slope ($\overline{b_{rel}}$), an additional one for calculating the ordinate intercept (a) and five more for finding the precision and accuracy.

In the transferable calibration factor (d), the factors f and f' are determined from known concentrations of internal standard (c_{is}) and standard (c_s) as in eqn. 2. Several plates were made and the mean factor $\bar{f}$ was determined. With this factor and the same amount of internal standard, applied with the unknown, the concentration (c) of the unknown is determined:

$$c = \bar{f} \cdot \frac{A}{A'} \tag{9}$$

where A = area of the unknown and A' = area of internal standard, applied with the unknown.

RESULTS AND DISCUSSION

Amounts of 10, 20, 30, 40 and 50 μg of diazepam were applied to the plates and evaluated. The correlation coefficients, the regression line equations and the calibration factors are given in Tables II and III. For selection of an internal standard, several benzodiazepines were tested; oxazepam was the most suitable because of its spectrophotometric and chromatographic behaviour.

The results of precision and accuracy in the limits of concentrations studied are given in Figs. 1 and 2. In the internal or direct calibration, the introduction of internal standard improves the precision and accuracy. In the external or transferable calibration, the introduction of internal standard improves the precision values while the accuracy values were not satisfactory except for the transfer of relative slope method. The methods with calibration factors were the most precise and accurate; the highest precision was found when the ratio of the areas was near to unity. The

TABLE II

CORRELATION COEFFICIENTS AND REGRESSION LINE EQUATIONS FOR METHODS OTHER THAN INTERNAL STANDARD METHODS

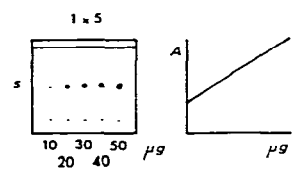
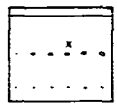
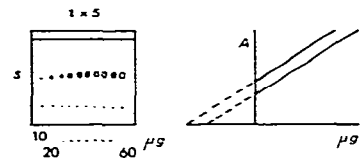
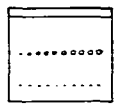
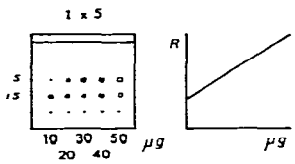
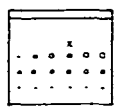
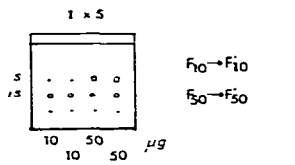
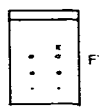
Method	$r (P < 0.001)$	y
A1a	0.9996	$0.67x - 11.5$
	0.9989	$0.71x - 10.1$
	0.9991	$0.70x + 11.2$
	0.9989	$0.74x - 10$
	0.9993	$0.71x + 10.5$
A1b	0.9991	$0.70x + 11.2$
	0.9992	$0.69x - 17.5$
	0.9996	$0.67x + 11.5$
	0.9995	$0.68x + 17.4$
	0.9995	$0.68x - 11.4$
	0.9992	$0.69x + 18.5$
	0.9991	$0.70x - 10.2$
	0.9992	$0.69x + 18.1$
	0.9996	$0.72x + 9.2$
	0.9989	$0.71x + 18.1$
B1a	0.9996	$0.706x + 10.66$
B1b	—	$0.706x - 10.82$
B1c	—	$0.704x + 10.88$

precision and accuracy of both methods were almost the same, although slightly better in those of internal calibration. The data pair technique could not be applied to higher concentrations. The lines had good correlation coefficients, but they are not parallel. When this method was applied to lower concentrations the precision

TABLE III

CORRELATION COEFFICIENTS, REGRESSION LINE EQUATIONS AND CALIBRATION FACTORS FOR INTERNAL STANDARD METHODS

Method	$r (P < 0.001)$	y	$f(10)$	$f(50)$
A2a	0.9985	$0.0225x + 0.385$	—	—
	0.9996	$0.0248x + 0.306$	—	—
	0.9998	$0.0233x + 0.361$	—	—
	0.9994	$0.0237x + 0.359$	—	—
	1.0001	$0.0234x + 0.358$	—	—
A2b	—	—	1.66	3.26
	—	—	1.65	3.19
	—	—	1.66	3.26
	—	—	1.66	3.22
	—	—	1.66	3.29
B2a	0.9999	$0.0239x + 0.353$	—	—
B2b	—	$0.0235x + 0.355$	—	—
B2c	—	$0.0233x + 0.361$	—	—
B2d	—	—	1.66	3.24

METHOD	CHROMATOGRAMS	EXAMPLE	μg/spot	Precision Sr	Accuracy
A1a			10 50	4.27 0.80	−1.70 −0.46
A1b			10 50	16.74 –	4.50 –
B2a			10 50	3.83 0.80	1.90 0.24
B2b			10 50	1.42 0.98	−1.20 −0.48

s = standard ; is = internal standard ; x = analyte ...

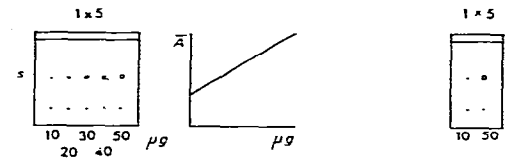
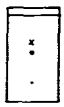
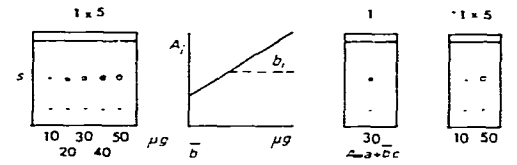
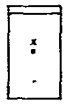
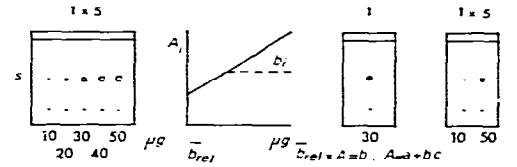
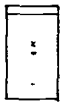
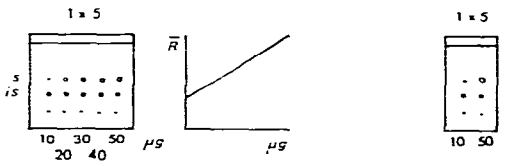
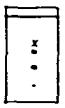
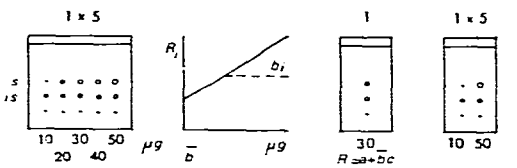
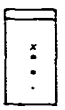
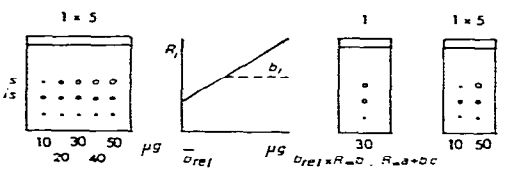
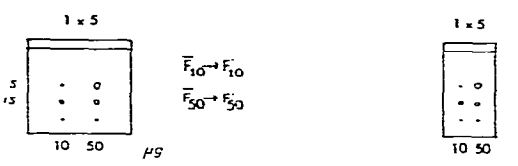
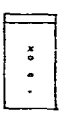
Fig. 1. Representation of densitometric methods. Internal or direct calibration.

and accuracy values were very poor; perhaps the introduction of an internal standard would improve the results.

In general, the results with internal calibration are better than the corresponding ones with external calibration, except when an internal standard is used which gives improved results for external calibration. With better instrumentation the sensitivity limits could be improved.

For spotting, the method of Shellard[17] was followed; it has been carried out using variable volumes corresponding to the concentration of each spot, although that increased the error. Plates prepared by us have been used; standard plates would probably give better results.

The work has been conducted in the most simple way. An inexpensive densitometer which operates only by transmission was used. The results would be greatly improved with instrumentation giving simultaneous measurements by transmission and reflection or remission[18]. For the measurement of peak areas corresponding to developed spots, the geometric[19] and planimetric methods were tested. The measurements with the planimeter always gave more reproducible values. If the peaks are perfectly regular, the geometric measurements gave good results.

METHOD	CHROMATOGRAMS	EXAMPLE	μg/spot	Precision S_r	Accuracy
B1a			10 50	6.23 2.37	1.10 −0.46
B1b			10 50	6.37 2.60	−1.20 −0.80
B1c			10 50	6.41 2.40	−1.80 −0.80
B2a			10 50	3.84 1.41	1.60 −1.18
B2b			10 50	3.71 1.41	2.50 0.34
B2c			10 50	3.76 1.43	0.90 0.68
B2d			10 50	1.51 1.08	−1.10 −0.60

s = standard; IS = internal standard; x = ...

Fig. 2. Representation of densitometric methods. External or transferable calibration.

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