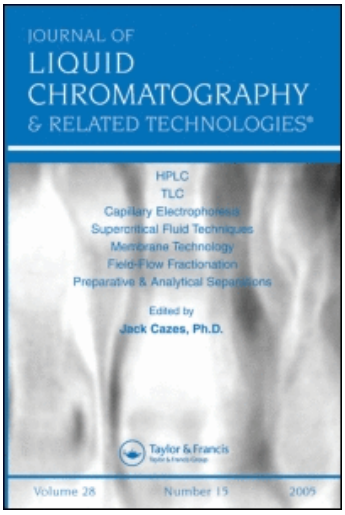

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PRACTICAL APPROACH FOR VALIDATING THE TLC ASSAY OF AN ACTIVE INGREDIENT IN A PHARMACEUTICAL FORMULATION

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ABSTRACT

A general approach is proposed for validating the TLC assay of an active ingredient in a dosage form. The experimental design together with the statistical tests used to evaluate the data takes into account the major aspects of the technique.

The present study is part of a work related to the experimental and statistical approach for validating an assay procedure of an active component in a dosage form. We had previously reported the possibility of using different statistical approaches on the basis of spectrophotometric data (1). This paper deals with the validation aspects of a TLC assay for the same active ingredient (diclofenac sodium) in the same tablet formulation. The proposed design and its statistical interpretation take into account the main features of the technique.

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PRELIMINARY EXPERIMENTS

There are no literature references concerning the TLC separation of diclofenac sodium (D) and its related decomposition products (Fig.1). The solvent mixture of dicloromethane-methanol in the ratio 92:8 (v/v) was found to be able to separate D from these impurities and to give an appropriate peak shape (Fig.2) and Rf value for quantitation.

Methanol was used as extraction solvent because it gave a satisfactory repeatability of loadings with the spotting device employed.

The on-plate maximum absorbance wavelength of D, 280 nm, was selected for the densitometric measurements.

EXPERIMENTAL

Apparatus, materials and chemicals.

A band applicator (Camag Linomat IV), HPTLC precoated silica gel plates 20x10 cm (Merck 60 F254) and a twin-trough chamber (Camag) were used for chromatographic development. Quantitative measurements were carried out with a chromatogram densitometer (Camag TLC Scanner II) coupled with an integrator (Merck-Hitachi D-2000).

All the chemicals were of analytical grade; D, decomposition products and commercial Voltarene L.P. tablets (100 mg of D for a tablet weight of 299 mg) were gifts from Ciba-Geigy laboratories (Basel, Switzerland).

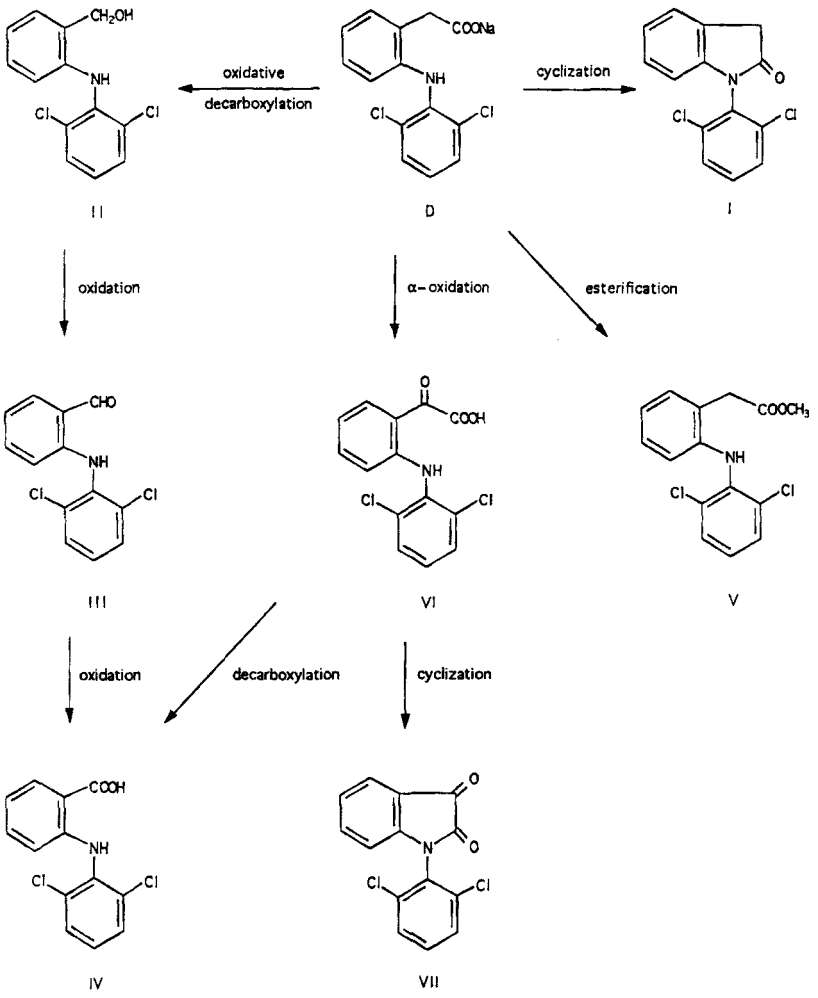

FIGURE 1. Decomposition products of diclofenac sodium.
D: diclofenac sodium
I: 1-(2,6-dichlorophenyl)oxindole
II: 2-[(2,6-dichlorophenyl)amino]benzyl alcohol
III: N-(2,6-dichlorophenyl)anthranililaldehyde
IV: N-(2,6-dichlorophenyl)anthranilic acid
V: 2-[(2,6-dichlorophenyl)amino]phenylacetic acid methyl ester
VI: {2-[(2,6-dichlorophenyl)amino]phenyl}glyoxylic acid
VII: 1-(2,6-dichlorophenyl)isatin

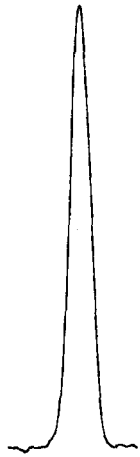

FIGURE 2. Chromatogram of a diclofenac sodiumstandard solution : 200 ng loading.

Test procedure.

-Solution for resolution test.

Prepare a stock solution (50 mgl^{-1}) of IV as follows: in a 100 ml volumetric flask, weigh accurately 5 mg of IV and add about 90 ml of methanol; dissolve by sonication and add methanol to the mark.

Prepare the solution for resolution test as follows: in a 50 ml volumetric flask, weigh accurately 50 mg of D and add 5 ml of the stock solution of IV and about 40 ml of methanol; dissolve by sonication and complete with methanol to the mark.

Prepare in triplicate the following solutions:

-Test solution.

In a 100 ml volumetric flask, place one tablet with about 90 ml of methanol, sonicate for 10 min. Allow to cool and add methanol to the mark. Centrifuge the suspension at 4000 rev/min for 30 min. Dilute 1/25 an aliquot of the supernatant with methanol to give a theoretical concentration of 40 mgl^{-1} of D.

-Standard solution.

In a 100 ml volumetric flask, weigh an accurate amount of 100 mg of D; add about 90 ml of methanol; dissolve by sonication for 5 min. Allow to cool and add methanol to the mark. Dilute 1/25 an aliquot of this solution with methanol to give a theoretical concentration of 40 mgl^{-1}.

-Sample application.

Pre-wash the plates by immersion for 1 h in methanol, dry for 30 min at 80°C. Allow to cool in a dessicator before use. Apply in triplicate 5 µl of standard and test solutions onto the plate using a bandwise applicator, at a delivery speed of 1 µl/10 s and as bandlengths of 5 mm. The space between bands and the distance from the side edge are determined by the number of samples to be analyzed.

-Chromatography.

Pre-equilibrate the plate in a twin-trough chamber (20x20 cm) with the vapors of the mobile phase dichloromethane-methanol (50 ml) in the proportion 92:8 (v/v) for 1 h. After this time, start the development and allow the mobile phase to migrate to a distance of about 60 mm which corresponds to a migration time of about 10 min. The spots can be visualized under UV light at 254 nm.

-Densitometry.

Perform quantitative measurements at 280 nm in the reflectance mode using a scanning densitometer with the parameters set as follows : monochromator bandwidth 10 nm, slit dimension 0.4x3 mm, scanning speed 0.3 mm s^{-1}. By using peak area measurements, calculate the percentage of D per tablet.

The results can be considered as valid if the performances of the chromatographic system meet the following

requirements : the Rf values for D should be about 0.27. The resolution between D and compound IV should not be lower than 0.70. The repeatability between three loadings should not be higher than 3%.

Test procedure validation.

-On-plate stability and solution stability.

A standard solution (40 mgl^{-1}) of D was applied on a plate in triplicate. After 1 h, the same solution was applied in triplicate on the same plate and the plate was immediately developed . The responses were compared.

The solution stability was assessed by loading a volume of 5 μl of a 24 h aged (at ambient temperature) and a freshly prepared standard solutions on the same plate. The response factors were compared.

-Specificity.

The interference of the related compounds and excipients of the formulation was assessed by applying 5 μl of a methanolic solution (40 mgl^{-1}) of each compound and of solutions resulting from the treatment of an analytical placebo and a placebo stressed in thermal conditions (60°C, for 7 h).

-Linearity and accuracy.

The linearity of the response of standard solutions was assessed by applying volumes of 3, 4, 5, 6 and 7 μl of the standard solution of D in triplicate on a same plate. These volumes correspond to a span of 60-140% of the theoretical content of D.

The linearity and accuracy of the test procedure were assessed by spiking five analytical placebos with D in solid form (60%, 80%, 100%, 120%, and 140% of the theoretical content). A single application of 5 μl of each test solution

was performed on each of three plates. The calibration line used to calculate the recoveries was obtained from two standard solutions (40 mgl^{-1}), each applied in volumes of 3, 4, 5, 6 and 7 µl on each plate. The average area of each pair was used to establish the regression equation.

-Repeatability.

The repeatability of the chromatographic system was assessed by applying onto the same plate six replicates (5 µl) of the standard solution of D. The RSD of peak area was calculated.

The repeatability of the test procedure was assessed by applying six times the procedure on real tablets and placebos spiked with 100% of the theoretical content. The amount of D was determined by reference to two standard solutions (40 mgl^{-1}), each applied in volumes of 3, 4, 5, 6 and 7 µl on the same plate.

RESULTS AND DISCUSSION

-On-plate stability and solution stability.

These tests should be carried out at the beginning of the validation process since they condition the validity of the other tests.

The on-plate stability should be assessed since on-plate degradation has been reported for some compounds (see e.g. ref.2). No significant difference ($p = 0.05$) was found between the responses obtained at one hour interval after the loading and no artefacts were formed within the limit of detectability of the method. D is stable on the plate for at

least one hour which is largely sufficient to cover the time necessary for band application. In addition, no significant difference ($p = 0.05$) was found in the densitometric measurements carried out at 90 min interval which allowed to cover a possible instrumental delay.

The comparison on a same plate between the response factors of solutions 24 hour aged at ambient temperature and freshly prepared were found unchanged ($p = 0.05$). This allows the solutions to be used within this delay without the results being affected.

-Specificity.

The solvent system dichloromethane-methanol 92:8 (v/v) allows the separation of D from compounds I-VII with respective Rf values of 0.27 and 0.38 for D and IV; compound VI does not migrate and other compounds are eluted near the solvent front.

-Linearity and accuracy.

* The linearity of the calibration curve (peak area *vs* applied amount) was first plotted on a graphic paper. A straight line not going through the origin was obtained (Fig.3). The regression line, calculated from the least-squares method was :

Peak area = (827.66 ± 38.35) applied amount (ng) + (58640 ± 8330) with the confidence intervals calculated at $p = 0.05$.

The coefficient of correlation was 0.997 and the coefficient of determination was 0.994. The variance analysis confirmed the linearity of the regression with a F_{cal} of regression = 2703 ($p << 0.01$) and F_{cal} of non-linearity = 2.06 ($p > 0.05$). The t-test showed that the regression line did not go through the origin ($t_{cal} = 15.21$), which should make

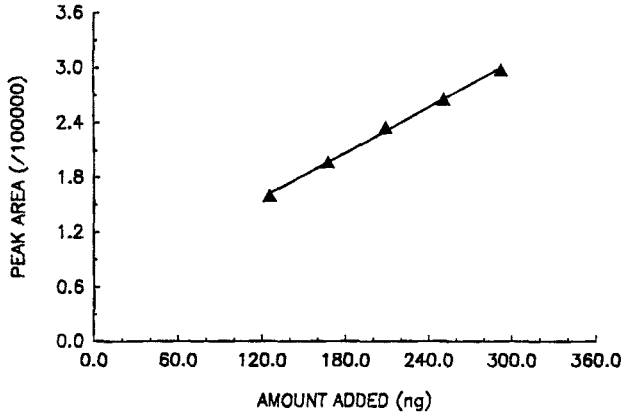

FIGURE 3. Calibration line for diclofenac sodium.

necessary to use multiple level calibration each time the procedure is carried out. However, since the test solution is at a target level, a single level calibration is possible; a dedicated software (3) may be of interest for the routine analysis in dosage forms.

*The linearity and accuracy of the procedure were investigated by repeating the chromatographic process on three plates (A, B and C) using the same standard and test solutions. This experimental approach takes into account plate-to-plate differences as well as other possible variations from one run to another (spotting, saturation, etc.). It gives an idea of the reproducibility (random errors) of the whole chromatographic system, in addition to accuracy (systematic errors).

The data were analyzed by using the three y-values (A, B and C) for each of the 5 x-concentration levels (TABLE 1) to construct the graph, after having assessed by one-way ANOVA on recoveries that there was no significant influence due to the plate.

The linear regression equation obtained was:

Amount found = (1.00 ± 0.04) amount added - (1.48 ± 7.51) at p = 0.05. The correlation coefficient was 0.998. ANOVA confirmed the linearity of the graph with F_{cal} of regression = 3092 ($p << 0.01$) and F_{cal} of non-linearity = 0.26 ($p >> 0.05$).

The resulting graph (amount found y - amount added x) should have a slope of 1 and go through the origin if the procedure is accurate and linear.

The t-test showed that the slope and intercept of the graph were not significantly different from 1 and 0 respectively ($p >> 0.05$). Therefore, the procedure proposed can be considered as linear and accurate in the range investigated. The general bias of the procedure given by the intercept corresponds to 0.73% of the value of y when x is at the 100% level. A bias of ± 2% can be accepted for the determination of an active ingredient in a pharmaceutical form (4). The experimental bias on each point (TABLE 1) is lower than 4% which is an acceptable value (5).

From the three experiments (A, B and C) the overall repeatability of recovery could be calculated (RSD = 1.87%, n = 15). The inter-plate reproducibility was found to be the same (RSD = 1.87%, n = 15).

- Repeatability.

The repeatability of the chromatographic system at the 100% level was 1.47% (6 replicate loadings on the same plate).

The repeatability of the test procedure was evaluated on real tablet samples and spiked placebos (100%) (n = 6 procedures). The diclofenac sodium content was calculated both by reference to the calibration line and the 100% calibration point (the results of which are given below in brackets).

TABLE 1. Linearity and accuracy of the test procedure.

Amount added (ng)	Amount found (ng)	Recovery (%)	Bias (%)
	123.9 (A)	101.06	1.06
122.6	118.0 (B)	96.25	-3.75
	118.9 (C)	96.98	-3.02
	165.2 (A)	99.76	-0.24
165.6	161.7 (B)	97.64	-2.36
	167.6 (C)	101.21	1.21
	204.3 (A)	98.79	-1.21
206.8	206.1 (B)	99.66	-0.34
	210.6 (C)	101.84	1.84
	243.0 (A)	98.94	-1.06
245.6	247.7 (B)	100.86	0.86
	242.8 (C)	98.86	-1.14
	272.5 (A)	96.70	-3.30
281.8	283.0 (B)	100.43	0.43
	283.9 (C)	100.75	0.75

For tablet samples, since the tablet weight is subject to small variations, each tablet was weighed before analysis and the percent of analyte recovered per tablet was also calculated with respect to the tablet theoretical weight. The diclofenac sodium content was found to be 99.05% (99.54%) for a tablet weight of 299 mg with a RSD of 2.93% (2.61%) which showed the repeatability of the procedure. The confidence interval on the assay result was 99.05% ± 3.05% (99.54% ± 2.73%). The amount of D per tablet was found to be 99.34% (99.83%) of the label claim with a RSD of 3.10% (2.77%). This result considers the variations of weight between tablets as well as the repeatability of the assay.

The recovery from spiked placebos was 98.89% (98.65%) with a RSD of 1.70% (1.55%). The confidence interval on the recovery was 98.89% ± 1.76% (98.65% ± 1.61%).

The results obtained by using either the calibration line or only a 100% calibration point do not give a significant

difference, which allows to use a single level calibration (at the targeted concentration) in routine, since the analyte is present in known amount.

CONCLUSION

It is often stated that the repeatability of TLC makes it more suited to impurity tests which are less demanding. However the results obtained in this study show that with optimized chromatographic conditions and an automated spotting device, the repeatability of the chromatographic system (RSD = 1.5%, n = 6) complies with the requirements for an active component assay in a formulation. It should be born in mind that a RSD of 2% (n = 5 or 6 injections) is generally admitted in the System Suitability Tests for HPLC (6).

The experimental design carried out to assess linearity, accuracy, reproducibility between plates and developments, as well as the statistical tests used to evaluate the data, shows that TLC is a reliable method for the determination of diclofenac in tablets. It can be used as an alternative method to HPLC for the assay of diclofenac sodium (7,8), especially for the analysis of a small number of samples.

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