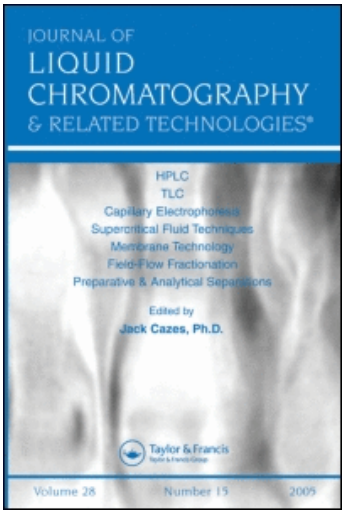

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Test Procedure Validation for the TLC Assay of a Degradation Product in a Pharmaceutical Formulation

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TEST PROCEDURE VALIDATION FOR THE TLC ASSAY OF A DEGRADATION PRODUCT IN A PHARMACEUTICAL FORMULATION

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ABSTRACT

An experimental and statistical approach for validating the TLC assay of a degradation product in a pharmaceutical formulation is given. This validation approach is intended to take into account the main particular aspects of the technique.

INTRODUCTION

We recently proposed a design for validating the assay of an active ingredient in a pharmaceutical formulation using spectrophotometry (1) and thin layer chromatography (2). UV spectrophotometry served as a basis for comparing various statistical approaches. Quantitative thin layer chromatography (TLC) illustrated the case of a complex technique in which the plate and mobile phase are renewed after each chromatographic development, the number of samples which can be analyzed in a same run is limited.

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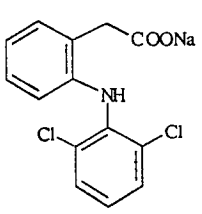

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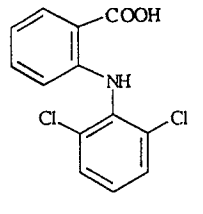

IV

FIGURE 1. Structure of Diclofenac sodium and compound IV used in the present study.

The present work concerns the particular aspects of a validation assay for an impurity using TLC with a special focus on the detection and quantitation limits (LOD and LOQ). At the LOQ, in addition to the accuracy and repeatability tests, a ruggedness test has been carried out to take into account the major sources of variations of the technique : the influence of some critical factors of the method (plate batch number, mobile phase composition and temperature) on the assay results, resolution and Rf values have been investigated.

The study has been carried out on one of the degradation products (see degradation scheme in ref. 2) of diclofenac sodium (D) in a tablet formulation. For the purpose of the work, the mobile phase used is such as the degradation product (compound IV, chosen as a model compound, Fig. 1) is located in the tailing of the active drug (present in large amount) as this illustrates a case which often arises in stability-indicating assays.

PRELIMINARY EXPERIMENTS

None of the solvent systems reported in the literature for D (3-6) could achieve a simultaneous separation of D and its degradation compounds. Different mobile phases have been investigated. The solvent system dichloromethane : methanol (92 : 8, v/v) allows the separation of D from IV with respective Rf values of 0.27 and 0.38, whilst compound VI does not migrate and other compounds are eluted near the solvent front (Rf > 0.83). Fig.2 shows the separation of IV at a 0.5 % level (with respect to D). Another solvent system, toluene : ethyl acetate (100 :3, v/v), should be used for the separation of the less polar compounds, which gives Rf values of 0.18 (II), 0.24 (I), O.29 (VII), 0.55 (V), 0.60 (III), while D, IV, and VI do not migrate.

Methanol was found to be the most suitable solvent both for extraction and on-plate application.

Quantitation was carried out at 280 nm, on-plate maximum absorbance wavelength of IV.

EXPERIMENTAL

Reagents, materials and apparatus.

All chemicals were of analytical grade. D, decomposition products and Voltarene L.P. tablets (containing 100 mg of D) were kindly supplied by Ciba-Geigy laboratories (Basel, Switzerland). For the chromatography, silica gel Merck 60 F 254, 20x10 cm HPTLC plates, a Camag twin-trough chamber (20 x 20 cm) and a Linomat IV (Muttenz, Switzerland) band applicator were used. A Camag TLC Scanner II chromatogram densitometer (Muttenz, Switzerland) with a deuterium lamp, connected to a Merck-Hitachi D-2000 integrator (Merck, Darmstadt, Germany) was used for densitometric measurements.

Test procedure submitted to validation.

-Standard solutions.

Use a reference batch of D free from compound IV .

Stock solution of D (2 g l^{-1}) : weigh accurately about 200 mg of D into a 100 ml calibrated flask, add about 90 ml of methanol, dissolve by sonication for 10 min, allow to cool and add methanol to the mark.

Stock solution of IV (50 mg l^{-1}) : weigh accurately about 5 mg of IV into a 100 ml calibrated flask, add about 90 ml of methanol, dissolve by sonication for 10 min, allow to cool and add methanol to the mark.

Working solutions (corresponding to 0.5%, 1%, and 1.5% of IV with respect to D) : place 25 ml of stock solution of D and respectively 5, 10, and 15 ml of stock solution of compound IV in three calibrated flasks of 50 ml. Make up to the mark with methanol.

-Test solution.

Place a tablet with about 90 ml of methanol in a 100 ml calibrated flask, sonicate for 10 min, allow to cool and add methanol to the mark. Centrifuge at 4000 rev min^{-1}. for 30 min. The supernatant is the test solution containing potentially compound IV.

-Sample application.

Before use, pre-wash the plates with methanol for 1 h, then dry at 80°C for 30 min and cool down. Apply in triplicate with the bandwise applicator 5 μl of each solution as bandlengths of 5 mm, at a delivery speed of 0.1 $\mu l\ s^{-1}$.

-Chromatography.

The chromatography should be carried out at about 20° ± 5°C.

Pre-equilibrate the plate with dichloromethane-methanol (92 : 8, v/v) vapors for 1 h. Then start the development and allow the eluent to migrate up to a distance of about 60 mm. The spots can be visualized under UV light at 254 nm : the respective Rf values are about 0.27 and 0.38 for D and IV. The resolution R, evaluated by the ratio a/b (Fig. 2) should not be lower than 0.70 at the 0.5% level.

-Densitometry.

Perform zero adjustment above the band of the analyte. Scan each track twice at 280 nm in the reflectance mode, using a 10 nm monochromator bandwith, a 0.4 x 3 mm slit dimension and a 0.3 mm s^{-1} scanning speed. Record the peak height. The repeatability between three loadings at 0.5% level should not be higher than 10%. Calculate the percent of compound IV in the tablet from the regression equation of the calibration line.

Test procedure validation.

Throughout the validation procedure, tablets were spiked using compound IV in methanolic solution. Unless otherwise stated, triplicate applications of a 5 μl volume of solution were performed as indicated in the routine test procedure.

-On-plate stability of the compound.

A standard solution containing 0.5% of IV (with respect to D) was applied on the plate; after 1 h the same solution was applied on the same plate and the plate was immediately developed. The response factors were compared.

-Stability of the solutions.

A standard solution (0.5% of IV) aged for 24 h at ambient temperature was applied on the plate. Its response factor was compared to that of a freshly prepared standard solution.

-Specificity.

The specificity of the technique for the determination of IV was tested by

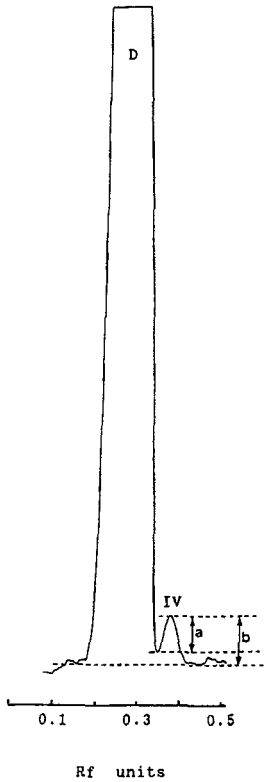

FIGURE 2. Chromatogram showing the limit of quantitation for compound IV (0.5% with respect to D).

applying the degradation compounds as indicated in the paragraph preliminary experiments. The non-interference of the formulation ingredients was assessed on an analytical placebo and a placebo stressed at 60°C for 7 h.

-Linearity and accuracy.

. Standard solutions.

The linearity of the response of the standard solutions was tested with five solutions containing 0.5%, 0.75%, 1%, 1.25%, and 1.5% of compound IV with respect to D (1 g l^{-1}) applied on the same plate.

Test solutions.

The linearity and accuracy of the test procedure were tested by spiking five tablets with compound IV (0.5%, 0.75%, 1%, 1.25%, and 1.5% with respect to D). The recovery was calculated by reference to the graph obtained from five standard solutions of the same concentrations, applied on the same plate. A single application of each test and standard solution was carried out on each of three plates issued from a same batch.

-Limit of detection.

The LOD was evaluated from a standard solution and subsequently confirmed on a tablet spiked at the same concentration (0.15% of IV) on plates from different batches and at either ends of the plates.

-Limit of quantitation.

The repeatability and accuracy of the test procedure at the LOQ were tested on six tablets spiked with 0.5% of IV (with respect to D). The recoveries were calculated from two series of standard solutions containing 0.5%, 0.75%, and 1% of IV (with respect to D). A single application of each solution was carried out on each of three plates issued from a same batch.

-Repeatability of the chromatographic system.

The repeatability of the chromatographic system was assessed by applying onto a same plate seven replicates of each of two standard solutions (0.5% and 1.5%).

-Ruggedness.

The ruggedness of the method was tested at the LOQ on a test solution prepared from a spiked tablet (0.5%); the recoveries were calculated by reference to a series of standard solutions (0.5%, 0.75%, and 1.0%). A Plackett and Burman experimental design was used (7). Each of the critical factors chosen (plate batch, mobile phase composition and developing temperature) was tested at three levels. The factor levels were as follows : three batch numbers; ratio dichloromethane : methanol (93 : 7, 92 : 8, or 91 : 9, v/v); temperature (15°C, 20°C, or 30°C). For the convenience of manipulation, the test was carried out in two blocks (higher + and basic 0 levels; lower - and basic 0 levels), each comprising four experiments.

RESULTS AND DISCUSSION

-On-plate stability.

It has been shown that some compounds are easily oxidized on the plate, the oxidation being catalyzed by the presence of metal ions in the silica coating, the acidity of the

silica, or the solvent system used (8). Therefore, the first test carried out was the assessment of on-plate stability of both the impurity and the active principle (present in large amount). The test was performed at the LOQ. No artefact was observed and no difference was found ($p = 0.05$) within the detection limits of the method, in the response factors for a solution applied at 1 hour interval. This delay allows to cover the time for band application. The on-plate stability of compound IV after development was also tested : no significant difference in the response was found between two scannings carried out at 90 min interval; this is sufficient to cover the measurement time. A two-dimensional chromatography was not carried out in this study because no degradation was noted during the chromatographic process; it should be performed if a degradation occurs during the chromatographic development.

-Solution stability.

The stability of the solutions containing 0.5% of IV was tested : no difference in the response factors was found ($p = 0.05$) between a solution aged for 24 h and a freshly prepared one. The solutions can be used within this delay without the results being affected.

-Specificity.

Under the conditions used, compound IV was well separated from the other degradation compounds and partially resolved from D. No interference of the formulation ingredients was observed.

-Linearity and accuracy.

Linearity of the calibration curve.

The test procedure uses standard solutions containing increasing amounts of IV in the presence of D (100% of the theoretical content) to imitate tablet solutions. The addition of the parent compound is particularly recommended when the impurity is located in the tailing of the parent compound as it allows the on-plate separation to be checked and a similar mode of measurement to be performed for standard and test solutions. In addition, it takes into account a possible change in the Rf values of the degradation product at trace levels in the presence of a large amount of the active drug. The linearity should be assessed from the LOQ to 150% of the tolerated limit (taken as 1% with respect to D for the purpose of the work).

The linearity of the calibration curve (peak height *vs* applied amount of IV) was first examined by a graphic plot : a straight line not passing through the origin was obtained (Fig.3).

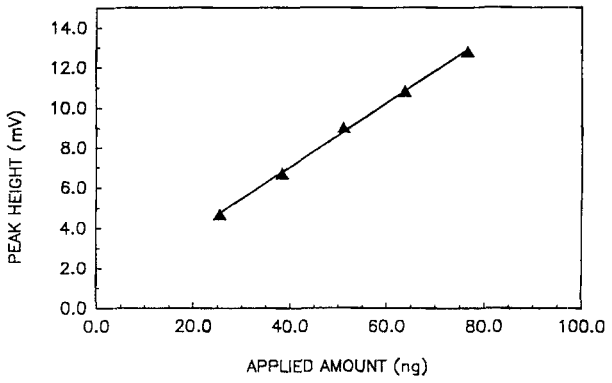

FIGURE 3. Calibration line for compound IV.

The regression line, calculated from the least-squares method was :

Peak height (μV) = (159.503 $\pm$ 6.070) applied amount (ng) + (698.933 $\pm$ 328.361), with the confidence intervals calculated at p = 0.05.

The correlation coefficient was 0.998. The linearity of the regression was assessed by ANOVA with F_{cal} of regression = 3221 ($p < 0.01$) and F_{cal} of non-linearity = 2.36 ($p > 0.05$).

Due to the fact that the regression line did not pass through the origin and that the analyte was not at a target concentration, a multi-level calibration was needed on each plate. For routine analysis, a graph constructed with three concentration levels was found to be sufficient.

Linearity and accuracy of the test procedure.

It should be noted that throughout this study, tablets were spiked with compound IV in solution. This can be accepted (9) when the analyte is not available in sufficient amount (in solid form) to prepare homogeneous samples simulating degraded tablets. In addition, it was assumed that by using a simple extraction procedure in a non-complex matrix together with a solvent having a high solubility *vs* the compound of interest, the major source of variations could be ascribed to the technique itself (sample application, plate-to-plate variations, development and densitometric evaluation). The proposed experimental approach is intended to take into account these variations by performing a single application of each solution on each of three plates, which

TABLE 1. Linearity and accuracy of the test procedure.

Amount added (ng)	Amount found (ng)	Recovery (%)	Bias (%)
	24.69	96.82	-3.18
25.50	23.99	94.08	-5.92
	26.75	104.90	4.90
	36.63	95.76	-4.24
38.25	35.78	93.54	-6.46
	37.95	99.22	-0.78
	51.90	101.76	1.76
51.00	49.21	96.49	-3.51
	49.66	97.37	-2.63
	67.19	105.40	5.40
63.75	62.17	97.52	-2.48
	62.60	98.20	-1.80
	79.13	103.43	3.43
76.50	71.60	93.59	-6.41
	78.29	102.34	2.34

allows the chromatographic system reproducibility to be evaluated. The results are given in TABLE 1.

The maximum experimental bias on each point was lower than 7%; a bias of ± 10% can be largely accepted for an impurity at these levels.

Linearity and accuracy were assessed by plotting the graph of amount found *vs* amount applied resulting from the three experiments. As a Barlett test showed that the recovery dispersions were not significantly different, one-way ANOVA was carried out on the recovery data. No significant difference between the plates ($p = 0.05$) was found and the graph was constructed using for each added amounts X the data from the three plates Y. The linear regression equation obtained was :

Amount found = (1.016 ± 0.068) amount added - (1.335 ± 3.709) with confidence intervals calculated at $p = 0.05$. The correlation coefficient was 0.994. The linearity was assessed by ANOVA with F_{cal} of regression = 837 ($p < 0.01$) and F_{cal} of non-linearity = 0.02 ($p > 0.05$). The t-tests showed that the slope of the line was not significantly different from unity ($t_{cal} = 0.517$) and that the line passed through the origin ($t_{cal} = 0.777$) corresponding to $p > 0.05$. The procedure could be considered as accurate and linear within the range investigated.

From the three experiments, an estimation of the overall repeatability (RSD = 3.24%, n = 15) and inter-plate reproducibility of recoveries (RSD = 4.32%, n = 15) was calculated.

-Repeatability.

The chromatographic system repeatability was 2.57% and 1.88% (n = 7 loadings) at respective levels of 0.5 and 1.5% of compound IV. The scanning repeatability was found to be 0.16% (n = 7 scans).

-Limit of detection.

The LOD is usually defined as the amount of substance which gives a signal-to-noise ratio of 2 or 3. In the case of an analyte eluted in the tailing of a parent compound, the background due to this latter is the limiting factor together with the noise and the amount which gives visually a shoulder can be considered as the LOD. The LOD was estimated to be 7.5 ng of IV in the presence of 5 μg of D, which corresponds to 0.15% of IV with respect to D (Fig.4). This evaluation was confirmed with tablets spiked at this level, on different plates and at either ends of the plates. Increasing the applied volume of solution (from 5 to 8 μl) did not give a noticeable change of the LOD due to a correlated loss of resolution by spot diffusion.

-Limit of quantitation.

The LOQ was estimated by the amount of compound which gave a signal equal to about 3 times that of the LOD (about 0.5%). This limit was subsequently validated (repeatability and accuracy) by the analysis of six extracts from tablets spiked with compound IV at this level on each of three plates (Fig.2). The recoveries are given in TABLE 2.

As the Bartlett test was not significant ($X^2 = 0.37$), one-way ANOVA was carried out which showed no significant effect of the plates on the results. The total mean recovery from the three plates was (98.98 $\pm$ 2.48)% with confidence limits on the RSD between 3.77% and 7.54%. The maximum experimental bias was 9.02%, which is largely tolerated at the LOQ. Although triplicate loadings are indicated in the test procedure for an accurate determination, the present data show that a single application of the solutions could comply with the assay requirements for a degradation product.

-Ruggedness.

In the United States Pharmacopeia (10), ruggedness is very similar to reproducibility. The ruggedness can be evaluated by studying the influence of critical factors of the

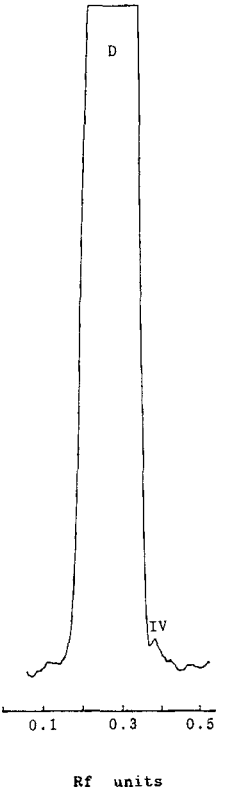

FIGURE 4. Chromatogram showing the limit of detection for compound IV (7.5 ng) in the presence of D (5 μg).

procedure successively or in combination through an experimental design (7). In the present study, selected factors were those which could be critical for separation, identification and quantitation : plate batch number, eluent composition and temperature. For quantitative factors, the variations used were in the range of order of those which could be found between laboratories. The influence of the different factors was tested at the LOQ on the assay, resolution (R) and Rf value, which are of major interest for identification and quantitative analysis. The results are given in TABLE 3.

A factor was considered as significant when the difference | D | between the average of results at extreme and nominal levels was larger than $\sqrt{2}$ times the SD at the nominal level (7, 11). Two-way ANOVA on reproducibility data for the procedure gave a SD of 5.69 and

TABLE 2. Repeatability and accuracy of the procedure at the limit of quantitation.

	Plate 1	Plate 2	Plate 3
	94.24%	97.25%	95.25%
	96.08%	96.35%	109.02%
	105.14%	99.10%	93.41%
	98.78%	104.55%	98.00%
	94.24%	108.20%	95.25%
	100.59%	96.35%	99.84%
m_i	98.18%	100.30%	98.46%
sd	4.24%	4.95%	5.65%
m		98.98%	
SD_r		4.98%	
SD_R		4.98%	

SD_r : SD of overall repeatability
SD_R : SD of inter-plate reproducibility

0.07 for assay and R respectively. Moreover, the SD between the Rf values of three plates was 0.01.

From TABLE 3, it can be seen that the plate batch number is only significant on the Rf (at a minor extent) and that a change in the mobile phase composition or temperature influence both resolution and Rf. However none of these factors has a significant effect on the assay results.

-Sensitivity.

It is commonly expressed as the slope of the calibration line but can also be assimilated to the resolution power of the method, i.e. the minimum variation of concentration or amount which gives a significant variation of the response with α and ß risks (9) :

Sensitivity = $(t_{(1-\alpha/2)} + t_{(1-ß)})\, s\sqrt{2}/b$, where b is the slope of the calibration line, s the standard deviation of repeatability and t the Student t-value. The sensitivity was found to be 7.25 ng at LOQ and 6.13 ng on the whole range of linearity (α = ß = 0.05). These results are very close to the limit of detection.

TABLE 3. Results of the ruggedness test.

NOMINAL LEVEL(0) AND LOWER LEVEL(-)

Exp.	Factors			Results			
	a	b	c	Assay	R	Rf	
1	0	0	0	93.73%	0.96	0.38	y_1
2	-	0	-	92.80%	0.82	0.46	y_2
3	0	-	-	94.13%	0.98	0.36	y_3
4	-	-	0	94.07%	1.00	0.32	y_4

Differences due to each factor for the respective results (%, R, Rf) :

Da = $[(y_1+y_3)-(y_2+y_4)] / 2$ = 0.50, 0.06,-0.02
Db = $[(y_1+y_2)-(y_3+y_4)] / 2$ = -0.84,-0.10, 0.08
Dc = $[(y_1+y_4)-(y_2+y_3)] / 2$ = 0.44, 0.08,-0.06

NOMINAL LEVEL(0) AND UPPER LEVEL(+)

Exp.	Factors			Results			
	a	b	c	Assay	R	Rf	
1	0	0	0	104.43%	0.98	0.40	y_1
2	+	0	+	97.14%	0.76	0.39	y_2
3	0	+	+	94.85%	0.62	0.42	y_3
4	+	+	0	101.53%	0.82	0.47	y_4

Da = $[(y_1+y_3)-(y_2+y_4)] / 2$ = 0.31, 0.01,-0.02
Db = $[(y_1+y_2)-(y_3+y_4)] / 2$ = 2.60, 0.15,-0.05
Dc = $[(y_1+y_4)-(y_2+y_3)] / 2$ = 6.99, 0.21, 0.03

CONCLUSION

This paper was not intended to cover all the aspects of TLC validation for a degradation product but to point out some particular aspects related to the determination at trace levels. Particular attention has been paid to some critical factors which may affect the results : within-batch and inter-batch plate variations, modification of the mobile phase composition and temperature. Other factors could be also relevant to study (applied volume, saturation time of the chamber, etc.). It should be noted that the proposed design is complicated by the fact that a multi-

level calibration is needed since the calibration graph does not go through the origin and the level of analyte is not assumed to be known.

TLC is mainly used in pharmaceutical industry as a qualitative or semi-quantitative method. However, this study shows that it gives reliable results for analytes at low levels and may be a useful alternative to HPLC (12), in particular when a small number of samples has to be analyzed.

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