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COMMUNICATION

Stability Study of Hard Gelatin Capsules Containing Retinoic Acid

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ABSTRACT

Retinoic acid (RA) is employed in the therapeutic treatment of acute promyelocytic leukemia (APL). In this paper, the chemical stability and the most favorable storage conditions of RA in hard gelatin capsules containing α-lactose monohydrate, used in clinical experimentation, are reported. A secondary goal of this work was to show the usefulness of a robust regression technique, repeated median with replicates (RMWR) in a solid-state shelf life prediction by accelerated studies. The capsules were stored at room temperature and in the freezer. Their residual RA content was assayed for more than 3 years. RA chemical degradation was monitored by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) stability-indicating methods previously validated and able to detect various potential degradation products. Possible physical modifications were checked by dissolution tests and differential scanning calorimetry (DSC) of the content of the capsules. The shelf life was also predicted by an accelerated isothermal method to confirm room temperature results, and the activation energy estimated through this study was 12.5 ± 1.1 kcal/mol (95% confidence interval). In the conditions of climatic zone II, the shelf life for the capsules stored at room temperature in light-resistant containers was equal to 678 days, while the capsules stored in the freezer retained the initial content of drug after 1289 days. From the results gathered in this study, the usefulness of RMWR for shelf life prediction in the presence of outliers is evident.

Key Words: *Retinoic acid; Robust regression; Shelf life; Stability.*

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INTRODUCTION

Retinoic acid (1) (RA) or Tretinoin is an endogenous retinoid that plays a key role in the normal differentiation and maintenance of the differentiated state of many epithelial tissues. A considerable body of evidence has been published on the ability of RA to influence the development of some tumors (2,3).

For these reasons, RA is considered a model drug for the differentiation therapy of neoplastic diseases. RA shows a remarkable therapeutic efficacy in the treatment of acute promyelocytic leukemia (APL), for which about 85% of patients achieve complete remission. After a variable period of complete remission, they relapse, showing resistance to subsequent treatments (4,5). Various mechanisms to explain the increased disappearance of RA from plasma and various pharmaceutical and clinical strategies to overcome the problem have been proposed (6,7). The encouraging therapeutic effect of RA in APL and its possible future use in other diseases has stimulated our group to undertake a more accurate investigation of biopharmaceutical and physicochemical properties of RA to design new dosage forms able to overcome the resistance and the adverse effects of the drug (8).

With the aim to have a simple comparison form to study the relative bioavailability and stability of different oral dosage forms, we propose a hard gelatin capsule containing 10 mg of RA mixed with Tablettose® (Meggle, Wasserburg, Germany), a physically well-characterized lactose, as the diluent.

In this work, we evaluate the shelf life t_{90} of these capsules stored at room temperature (RT) and in the freezer (FR). The RA chemical degradation was monitored by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) stability-indicating methods previously validated (9,10). The methods were able to detect various potential degradation products. Possible physical modifications were checked by dissolution test (DT) and differential scanning calorimetry (DSC) of the content of the capsules. To confirm RT results and simultaneously test the robust regression technique RMWR (11), we also estimated t_{90} by an accelerated isothermal degradation method. The kinetic hypothesis of RA solid-state decomposition and some thermodynamic values were calculated and compared to those found in the literature.

EXPERIMENTAL

Materials and Instruments

RA was supplied by Istituto delle Vitamine S.p.a. Roche (Milan, Italy). Tablettose Meggle was supplied by Giusto Faravelli S.p.a. (Milan, Italy). White hard gelatin capsules, Coni-Snap™ no. 3, were supplied by Capsugel Parke Davis S.p.a. (Milan, Italy). Acetonitrile and water were HPLC grade, and all other chemicals and solvents were analytical grade and were used as supplied. The HPLC system consisted of an HP1090 equipped with diode array detection (DAD) (Hewlett Packard, USA). The TLC plates were of Kieselgel 60 F254 supported by aluminum (Merck, Bracco S.p.a., Milan, Italy). The DSC apparatus was a DSC-7 managed by a standard DSC-7 software program (Perkin Elmer, Norwalk, CT). The Erweka motor AR400 (Heusenstamm, Germany) was equipped with a small (120 ml) home-made cubic tumbling mixer. The mechanical convection ovens were Gallenkamp 300 Plus series. The dissolution tester was Sotax AT7 (Allschwil, Switzerland) managed by an automated dissolution testing software for the HP8452A UV DAD spectrophotometer.

Capsule Preparation

The RA used was sieved to obtain a powder with a particle size smaller than 250 μm. A 5.7% ± 0.5% (w/w) mixed powder of RA in Tablettose was prepared by mixing the ingredients for 4.5 hr in the mixer at 60 rpm. During the mixing, the uniformity was monitored by HPLC assay of accurately weighed samples of powder equivalent to about 10 mg of RA. The uniform powder was packed in the capsules by a manual filling machine.

High-Performance Liquid Chromatography Assay

The column was a 250 × 4 mm HPLC Cartridge LiChrospher® 100 RP-18 (5 μm) (Merck, Darmstadt, Germany). The mobile phase was composed of 90% (v/v) acetonitrile and 10% (v/v) of 1% (w/v) ammonium acetate aqueous solution mixed by the HPLC pump. The injection volume was set at 10 μl. The flow rate was set at 1.250 ml/min. In these conditions, the RA retention time t_R was about 9 min. The analytical DAD wavelength was set at 340 nm with a bandwidth of 4 nm, and the reference wavelength was at 500 nm with a bandwidth of 80 nm. The RA standard and the sample solutions were prepared, as described in Ref. 9, to obtain a solution having a known concentration of about 0.2 mg/ml.

Thin-Layer Chromatography Conditions

The developing solvent consisted of chloroformmethanol (90:10) containing 5 mg/ml of BHT (butylated hydroxytoluene). Every silica gel plate was dipped into a

BHT solution in diethyl ether (5 mg/ml) and left to dry in the air. The RA standard solution was prepared at a concentration of 10 mg/ml; the other standard solutions for identification of possible degradation products were prepared at a concentration of 1 mg/ml. The application volume was 10 μl, and the development distance was about 10 cm. The sample preparation and the detection of spots are described in Ref. 10.

Differential Scanning Calorimetry

The apparatus was calibrated with indium and zinc. An amount of the content of a capsule, ranging from 4.5 to 5.5 mg, was accurately weighed in an aluminum sample pan and closed by a handled crimper. Samples were scanned at the rate of 10°C/min in the range from 50°C to 250°C. The holder was continuously purged by about 20 ml/min nitrogen flow. The thermal events were described using the extrapolated onset temperature.

Dissolution Test

The USP apparatus 2 (paddle, stirring rate 100 rpm) was used. The test was performed at 37°C ± 0.5°C on six capsules simultaneously using automatic equipment for sampling. The dissolution medium (550 ml) was prepared by dissolving 1% (w/v) of ammonium acetate and 0.8% (w/v) of sodium cholate and adjusting the pH to 7.4 ± 0.1 with a dilute ammonia solution. The amount of RA dissolved was automatically measured by UV/DAD absorbance (analytical wavelength 340 nm) every 10 min for 120 min. The RA dissolved (percentage based on the theoretical capsule content of 10.0 mg) was assayed with respect to the amount dissolved at 120 min determined in comparison with a standard solution (1:5 medium:methanol) having a known concentration (about 0.001 mg/ml).

Stability Studies Procedures

All operations were performed in a darkened room illuminated with yellow light. RA potency was expressed as a percentage (w/w) of the capsule content. Two batches of capsules, called A and B, were packaged in amber glass bottles closed by screw caps. Batch A was used to prepare bottles stored at 30°C, 40°C, and 50°C; batch B was for those stored at RT, at 60°C, and in the freezer. All the bottles were stored in the dark. As the RA working standard (WS), the same lot used to prepare the capsules was stored in 1-ml glass amber ampoules flame sealed under nitrogen atmosphere. The WS ampoules, each containing 40 mg of RA, were stored in the freezer. At every sampling time, a new WS ampoule was used in the various tests described above. Every year, the WS was reassayed against a new certified RA master standard. For each temperature considered, at the pull times indicated in the Results section, 10 or 3 capsules were sampled for HPLC, 3 for DSC and TLC, and 6 for DT. The lag time between the pull time (12) and assay or test time was no longer than 12 hr, and during this time, the samples were stored in the freezer. The RT extrapolation used was 25°C, as recommended for climatic zone II (13).

RESULTS AND DISCUSSION

In a preliminary study, RA was shown to be compatible with α-lactose monohydrate in a 50% (w/w) mixture if stored at 40°C and 80°C for 120 days and 14 days, respectively, at least. Moreover, the RA stability in this mixture was not influenced by the control of relative humidity. For this reason, we selected a special grade of α-lactose monohydrate as the capsule diluent. The good flow properties and small particle size distribution of this commercial product (Tablettose, Meggle) (14) allowed to obtain, in a short mixing time (4.5 hr), good RA content uniformity (RSD ≤2%) and weight uniformity (the average weight of a sample of 80 capsules was 227.5 mg, with RSD <2.5%) without the addition of other excipients. Two batches of capsules, packed in amber glass bottles, were stored at RT, in the freezer, and at four different stress temperatures (30°C, 40°C, 50°C, and 60°C). At each storage temperature, the physical and chemical stability of this dosage form was monitored by reversed-phase HPLC stability-indicating assay (9), NP-TLC (normal-phase thin-layer chromatography) (10), DSC, and dissolution rate profile. The RA content and its uniformity between two batches used in this study were not significantly different ($p > .05$). The RA residual potency of the capsules stored at RT for 3.5 years, at the various sampling times, is recorded in Table 1.

The main outcomes obtained by the application of ordinary least-square (OLS) regression to the RT stability data, according to zero-order and first-order kinetic models, are listed in Table 2.

Although the RA degradation was evident from the data of Table 1, the distinction between zero- and first-order kinetics was difficult to discern due to the following: (*a*) at the solid state, decomposition takes place in a heterogeneous complex system; (*b*) in solid dosage forms, the content uniformity is not as good as in solution dosage forms; (*c*) the duration of the study did not allow reaching a sufficiently large RA decomposition.

Table 1

Residual Retinoic Acid Potency of Capsules Stored at Room Temperature Expressed as a Percentage (w/w) of Content

Sampling Time (days)								
34	62	97	175	268	377	538	742	1289
5.61	5.42	5.57	5.45	5.54	5.52	5.22	5.15	4.64
5.80	5.66	5.70	5.55	5.35	5.58	5.46	5.27	4.53
5.49	5.70	5.39	5.52	5.70	5.42	5.20	4.95	4.62
					5.62	5.27	5.04	4.63
					5.24	5.17	5.06	4.66
					5.43	5.27	5.00	4.55
					5.41	5.34	5.20	4.68
					5.54	5.06	5.10	4.58
					5.61	5.37	5.05	4.62
					5.32	5.40	4.98	4.61

Examining the outcomes of Table 2, we considered that the difference between the squared multiple correlation coefficients R^2 (15) is not significant to estimate the reaction order, and the criterion to compare the least standard error of the estimate (SEE) cannot be applied. Even if the choice between the two considered reaction orders has a little influence on the t_{90} estimate (Table 2), a tentative assignment can be done on a statistical basis. In fact, as pointed out by Draper and Smith (16), the best mathematical model is the one explaining most of the total data variation. Since, as shown in Table 2, the percentage of model explanation (see Appendix) is slightly larger for zero order, we can hypothesize that the RA degradation kinetics follow this rate law. Further observations seem to confirm this supposition. First, the degradations at the accelerated temperatures (Fig. 1) follow more closely the kinetics of zero order; second, there is a good correlation in the Arrhenius plot between the accelerated zero-order constant rates and that obtained at RT; third, the microcalorimetric study reported by Tan and coworkers (17) established that the RA degradation, in the solid state under air atmosphere, follows a zero order.

Table 2

Comparison Between Ordinary Least-Squares Parameter Estimates Obtained from Stability Data Shown in Table 1 According to Zero and First-Order Kinetics

	Kinetic Order	
	Zero	First
R^2	.905	.909
SEE	0.115	0.022
Intercept	5.70	1.74
Slope	−8.27E-04	−1.61E-04
Sb	3.4E-05	6.4E-06
ln $\|k\|$	−7.09	−8.73
Maximum R^2 attainable	0.919	0.926
% of model explanation	98.5	98.2
F test lack of fit	0.36	0.16
t_{90} 95% one-sided confidence interval	678–715	662–697

R^2 is the squared multiple correlation coefficient; SEE is the standard error of estimate; *Sb* is the standard error of the slope; and k is the degradation rate constant.

For this rate law, t_{90} at RT, expressed as 95% one-sided confidence interval (CI), is equal to 678–715 days.

Before estimating by OLS the RA degradation rate constants k at the various stress temperatures, the data must be inspected and cleaned of possible outliers. These anomalous observations are typical of the accelerated study on the solid state. In our data, not many outliers were detected: 1, 3, 2, and 4, respectively, for 30°C, 40°C, 50°C, 60°C storage conditions.

To improve the precision of the expiration dating period, the robust regression method RMWR (11), suitable for data from stability studies, was employed on an uncleaned data set. This method is able to give trustworthy results even in the presence of a certain fraction of outliers. The difference between the estimates obtained by the two methods was evident from Fig. 1, in which the two lines are compared at the four stress temperatures.

The noticeable goodness of RMWR estimates with respect to OLS can be evaluated in Fig. 2, in which the two treatments are compared on the Arrhenius plot. In this plot, the values of k obtained by RMWR show better correlation and precision than those obtained by OLS. In fact, the best fitting of the line to experimental points makes SEE smaller and the extrapolation CI narrower: The one-sided CI at 25°C was 432–474 days by RMWR against 286–477 days by OLS.

The extrapolated RMWR k_{25} appears to underestimate the t_{90} found at RT. The seeming inconsistency of these data is due to the choice of adopting the climatic conditions suggested by the International Conference on Harmonisation (25°C) (13,18) for our latitude. In fact, if we extrapolate the t_{90} at 19°C, which is the more likely annual mean laboratory temperature (building without nightly thermal conditioning), the 95% one-sided CI becomes equal to 650–730 days.

Furthermore, the activation energy E_a calculated by RMWR Arrhenius plot is 12.5 ± 1.1 kcal/mol (95% CI). This value is in agreement with the less precise estimate reported in the paper of Tan et al. (17). The authors of

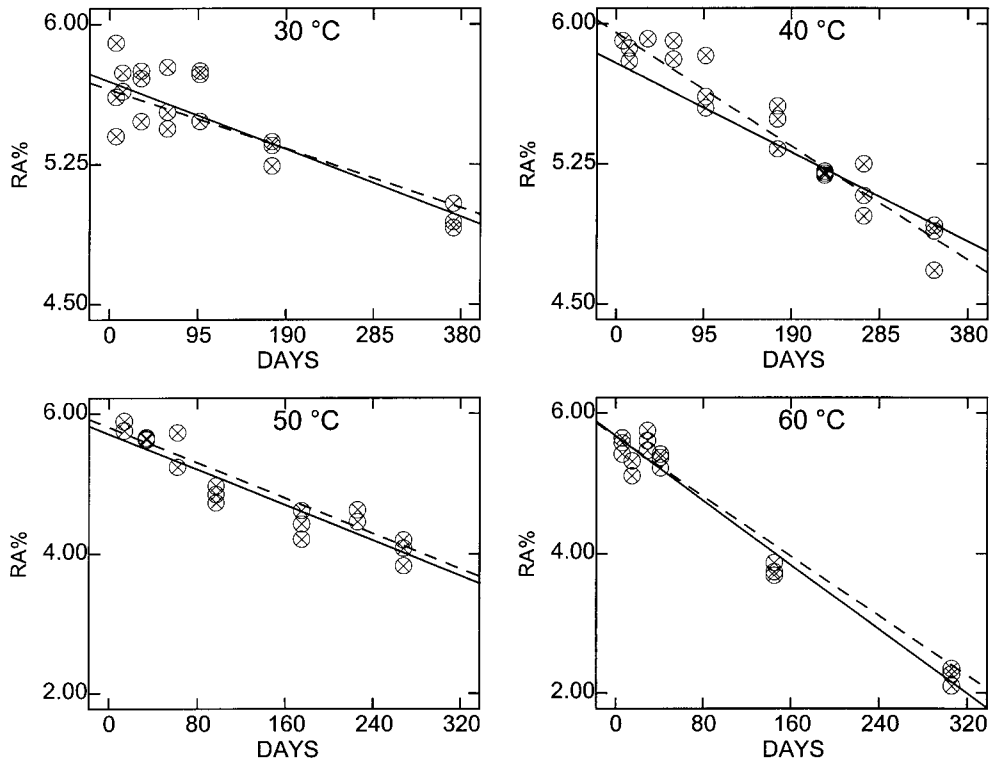

Figure 1. Percentage of residual retinoic acid (RA%) in capsules stored at four different temperatures (30°C, 40°C, 50°C, 60°C). For each temperature, the regression line by least square after outlier elimination (—) and that obtained by application of the robust regression RMWR on the whole stability data set (- - -) are superimposed.

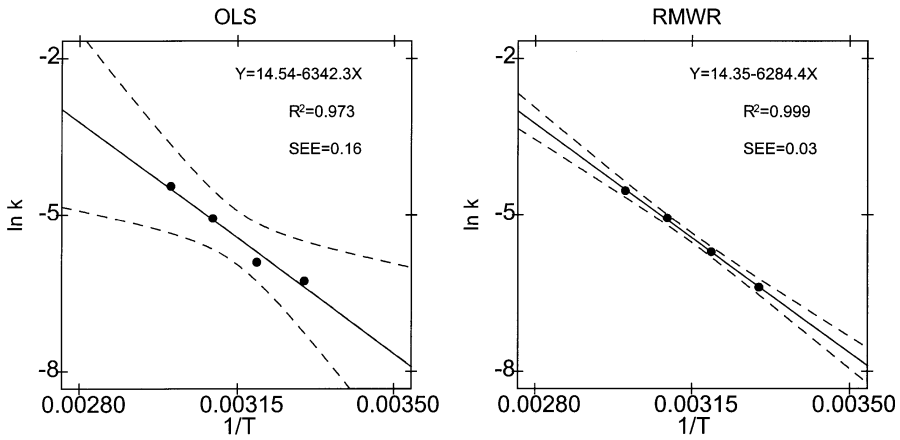

Figure 2. Comparison between Arrhenius curves obtained from degradation rate constant *k* by ordinary least-square (OLS) treatment and by robust regression (RMWR). The dashed lines represent the 95% confidence interval. R^2 is the squared multiple correlation coefficient, and SEE is the standard error of the estimate.

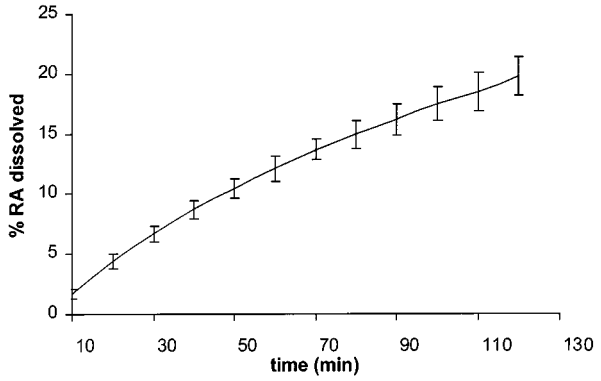

Figure 3. Typical capsule dissolution rate profile. Each point is the mean of results from 6 units, and vertical bars represent the standard deviation. For this profile, the value of t_{30}, t_{60} (%RA dissolved), and dissolution efficiency *DE* are, respectively: 6.7 ± 0.7, 12.1 ± 1, and 12.2 ± 1% (average ± standard error).

this work reported the E_a, expressing the uncertainty as the standard error of the slope of the Arrhenius line (13.9 ± 0.7 kcal/mol); considering that they used five temperatures to build the plot, their E_a estimate is equal to 13.9 ± 2.3 kcal/mol (95% CI).

The capsules stored in the freezer were stable for 3.5 years. In fact, the RA contents did not show significant reduction over the storage period with respect to initial assay (analysis of variance [ANOVA], $p > .05$).

During the study, the physical stability of the capsule contents was monitored by DSC and DT to point out every possible physical modification. A characteristic scan of the content of a capsule shows the following events: lactose dehydration endotherm (for 40 runs, 145.1°C ± 0.2°C average, 95% CI), RA melting endotherm (for n = 40 runs, 181.1°C ± 0.1°C average, 95% CI), α-lactose melting endotherm (for n = 40 runs, 209.1°C ± 0.3°C average, 95% CI), and lactose charring endotherm at about 221°C. The nature of these events was confirmed by hot-stage microscopy and thermogravimetric analysis. If RA degradation did not occur, no DSC thermal events, detected in the scan of the powder mixed just before encapsulation, underwent any modifications during the storage. Obviously, the RA signal decreased up to disappearance when the degradation exceeded the DSC detection limit (i.e., for the capsule stored at RT for 1289 days).

A robust DT developed for the stability studies was validated by the HPLC method described. A typical dissolution rate profile with relative parameters is plotted in Fig. 3. For each storage condition, if the RA content remained within 90% of the initial amount, the DT profiles were superimposable, and the principal comparative parameters, like t_{30}, t_{60}, and dissolution efficiency (19), did not show any significant difference (ANOVA, $p > .05$).

Although, when bottles containing degraded capsules were opened, a smell could suggest the formation of volatile compounds, no degradation products were pointed out during the study. The thin layers of the degraded samples showed light tailing spots running very slowly ($Rf < 0.1$), which could indicate the presence of some unidentified polar degradation products.

CONCLUSIONS

The evidence gathered in this study showed that the shelf life of RA capsules stored at room temperature in light-resistant containers is equal to 1.8 years. Although these data are also supported by accelerated stability studies, a more conservative shelf life settlement suggests that the use of the clinical batches, if suitably stored at room temperature, should not be extended beyond a year. The study also pointed out a shelf life of at least 3.5 years for the batch stored in the freezer; it follows that this is the best storage condition for the dosage form considered.

No degradation products were detected during the stability analysis, so further studies should be developed to elucidate the mechanism of the RA degradative reaction in the capsules described.

Moreover, in this paper, the goodness of the robust regression method RMWR in shelf life prediction, using accelerated stability data, was shown.

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APPENDIX

The percentage of model explanation (%*ME*) computed as

$$\%ME = \frac{R^{2\text{actually attained}}}{\max R^2} \quad (1)$$

where R^2 actually attained is the squared multiple correlation coefficient that measures the proportion of total variation explained by the fitted model, and max R^2 is the maximum R^2 attainable with the same data:

$$\max R^2 = \frac{\sum_{i=1}^{n} (Y_i - \bar{Y})^2 - \sum_{j=1}^{m} \sum_{u=1}^{n_j} (Y_{ju} - \bar{Y}_j)^2}{\sum_{i=1}^{n} (Y_i - \bar{Y})^2} \quad (2)$$

where Y_i is the ith observation, $\bar{Y}$ is the overall mean of the n observations, and Y_{ju} is the uth repeated observation ($u = 1, 2, \ldots, nj$) at X_j, where j are the sampling times.

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