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STABILITY INDICATING HPLC ASSAY FOR RETINOIC ACID IN HARD GELATINE CAPSULES CONTAINING LACTOSE AND AS BULK DRUG SUBSTANCE

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ABSTRACT

A reversed phase high performance liquid chromatographic assay for determination of Retinoic Acid in raw materials and in hard gelatine capsules containing Tablettose® as excipient is described. The method uses a UV diode array detector. The solution concentrations were measured on weight basis to avoid the use of an internal standard, otherwise necessary for the presence of the undissolved matrix. The study of the recoveries, planned in a Latin square as experimental design, shows that the accuracy and precision of the method are excellent. The assay is selective and stability indicating. The method resolves three related substances (13-cis-retinoic acid, 5,6-epoxy-5,6-dihydroretinoic acid and 4-oxo-retinoic acid) with quantitation in the range 0.04 ÷ 0.2 % of the labelled amount of Retinoic Acid (10 mg).

INTRODUCTION

Retinoic Acid (Tretinoin)[1] (RA) is an endogenous retinoid that is in the pathway of Vitamin A metabolism. Evidence gathered in these last years indicate that the role of RA is in the cellular differentiation[2]. Recently two classes of RA nuclear hormone receptors were identified[3]. While its potential use in the chemoprevention of cancer is restricted because of the low therapeutic index, RA is considered as model drug for the differentiation therapy of neoplastic diseases. Recent studies have shown that in high proportion of patients with Acute Promyelocytic Leukemia (M3 of FAB classification) (APL) complete remission with RA treatment is achieved. After a brief period of continuous complete remission they relapsed and showed resistance to subsequent treatment[4-6]. The resistance is associated with a progressive reduction of plasma concentration of RA to levels that may be inadequate to sustain differentiation in vivo[7]. This resistance can be due to many additional factors one of which may be an induced progressive impairment of gastrointestinal uptake[8]. The encouraging therapeutic effect of RA in APL and the future application in other diseases will stimulate the study of new dosage forms.

In order to study the gastrointestinal uptake and the relative bioavailability[9] of RA between different oral dosage forms, we propose, as simple comparison form, a hard gelatine capsule containing 10 mg of RA mixed with Tablettose®, a physically well characterized lactose, as diluent. For assaying and monitoring the stability of RA in this capsule a stability indicating[10]

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analytical method is necessary. Various methods have been reported for determining low concentrations of RA in biological media[11]. In this paper a reversed phase (RP) high performance liquid chromatography (HPLC) assay appropriate to detect and quantitate RA and its degradation products in this dosage form is described.

The method uses a UV-VIS diode array detector which allows simultaneous monitoring of a sample at different wavelengths for the search of degradation products, checking peak for purity and confirming peak identity[12].

The stability indicating ability was demonstrated only for thermal degradation conditions because the stability study was planned in the dark for different storage temperatures.

The selectivity of the RA assay makes possible the simultaneous detection and quantization of some related substances[13-14]: 13-cis-retinoic acid (CRA), 5,6-epoxy-5,6-dihydroretinoic acid (ERA), 4-oxo-retinoic acid (ORA) .

Validation studies to test the method for linearity, selectivity, stability indicating ability, accuracy, precision, system suitability, ruggedness have been carried out.

MATERIALS AND METHODS

Solvents and Chemicals

RA was supplied by Sigma Aldrich S.r.l., Milan Italy (Lot N°40h0313) and Istituto delle Vitamine S.p.a. Roche, Milan Italy (Lot N°904008). CRA (Lot N° 92H7701 and Lot N° 118 F0603) was supplied by Sigma Aldrich S.r.l., Milan, Italy. ORA (Ro 12-4824), and ERA (Ro 08-3249) were supplied by Istituto delle Vitamine S.p.a. Roche, Milan Italy. Tablettose® Meggle was supplied by Giusto Faravelli S.p.a., Milan Italy. Ammonium acetate pro analysi Merck, Acetonitrile LiChrosolv Gradient Grade Merck and Potassium hydroxide pellets p.a. Merck were supplied by Bracco S.p.a., Milan Italy. Methanol p.a. and Hydrochloric Acid (assay > 36.5% w/w) Fluka were supplied by Schrepfer S.p.A., Milan. Water HPLC grade was obtained by Milli Q System. White hard gelatine capsules, Coni-Snap™ N°3 were supplied by Capsugel Parke Davis S.p.a., Milan Italy. Helium (purity > 99.998%) was supplied by SIAD (Italy).

Instruments

The HPLC system consisted of a Pump and Solvent Delivery System HP 1050 Series equipped with a manual injector fitted with a 10 µL loop, a Diode Array Detector (DAD) HP 1040M managed by a HP 98572A Chem Station, equipped with the operating software HP 79995A Rev.4.22 and a Colorview software 79997A Rev.5.20, Hewlett Packard (USA). The ultrasonic dissolution bath was a Branson 2000 (Connecticut USA), working frequency 47 KHz ± 6 %. The Analytical Balance was a B120S Sartorius AG (Göettingen, Germany). The ultramicro balance was a AD-4 Autobalance Perkin Elmer (USA). The Erweka motor AR400 (Heusenstamm Germany) was equipped with a small (capacity = 120 mL) home made Plexiglas cubic tumbling mixer, with three Plexiglas diagonal baffles and closure sealed by polytetrafluoroethylene (PTFE) liner. The manual capsule filling machine was a MC2 Multigel (Florence, Italy). The other mixer was a model T2C Turbula W.A. Bachofen (Basel, Switzerland).The system to obtain water HPLC grade was a Milli-Q-plus Millipore (Molsheim France).The yellow light in the darkened room was obtained from a lamp Philux 60W Philips (Holland). The sample was filtered by a disposable syringe filter holder through a cellulose acetate membrane (pore size 0.45µm) Minisart NML 165555K Sartorius GmbH (Göettingen, Germany). Ammonium acetate HPLC solution was filtered under pressure by an equipment Sartorius SM 16249 through a cellulose acetate membrane (pore size 0.45µm) 11106-47-N Sartorius AG (Göettingen, Germany). For degradation studies, by heating under reflux, a double neck round bottom flask with a thermometer and a double coil condenser were used.

Chromatographic Conditions

The column was a LiChroCART® 250*4 mm HPLC Cartridge LiChrospher® 100 RP-18 (5 µm) Merck, coupled with a guard column LiChroCART® 4*4 mm LiChrosorb® RP-18 (5 µm)

Merck (Darmstadt, Germany). The mobile phase was composed of 90% (v/v) acetonitrile and 10% (v/v) of 1% (p/v) ammonium acetate aqueous solution, degassed with helium and mixed by the HPLC pump. The flow rate was set at 1.250 mL/min. (mean column pressure was 9.8 MPa). The run time was 16 minutes. The column was used at ambient temperature (22 ± 4 °C). In these conditions the RA retention time (t_R) was about 9 minutes. The analytical DAD wavelength was set at 340 nm with a bandwidth of 4 nm, and the reference wavelength at 500 nm with a bandwidth of 80 nm. The spectrum of every chromatographic peak with signal higher than 0.5 mAU was stored (resolution for 2 nm diodes).

Preparation of the RA Standard Solution

The solution, at the concentration of 0.25 mg/g (amount of solute/amount of solvent), was prepared as follows: to 5.0 ± 0.2 mg of RA weighed into a 25 mL volumetric flask, methanol was added to about the mark and its weight accurately determined. The resultant suspension was sonicated for 15 minutes, under periodic shaking.

Preparation of the Solutions Used in the Assay Validation

Linearity and Recovery Solutions for RA

For the study of the response linearity of RA, five methanolic solutions at concentration ranging from 0.12 mg/g to 0.38 mg/g were prepared by accurately weighing RA and sonicating the resultant suspension for 15 minutes, under periodic shaking.

For the RA recovery experiments three methanolic solutions at the concentrations of 0.10, 0.25 and 0.40 mg/g were prepared by spiking 166 ÷ 168 mg of Tablettose®, into 50 mL volumetric flasks, with 5.0, 10.0 and 15.0 mg of RA respectively. After mixing for 5 minutes in the Turbula mixer, the powders were extracted as described in Preparation of the Sample Solutions.

Linearity and Recovery Solutions for ORA, ERA and CRA

For the study of the response linearity for each related substances five solutions at concentration ranging from 0.1 μg/g to 3.0 μg/g were obtained by diluting appropriate aliquots of stock solutions with methanol.

For the recovery experiments a Recovery Stock Solution of ORA, ERA and CRA at the concentration of 13 μg/g, for each substance, in a RA Standard Solution was prepared. The Recovery Sample Solutions were prepared by spiking 166 ÷ 168 mg of Tablettose® into 50 mL volumetric flasks with 8 g of the Recovery Stock Solution and diluting to about the mark with an accurate weight of the same RA Standard Solution used for the Recovery Stock Solution. The resultant methanolic suspensions were subjected to the same procedure described in Preparation of the Sample Solutions. The standard solution used to evaluate the recoveries was prepared by weighing 8 g of the Recovery Stock Solution into a 50 mL volumetric flask and diluting to about the mark with an accurate weight of the same RA Standard Solution used to prepare the Recovery Stock Solution.

Capsules Preparation

A 5.7 ± 0.5% (w/w) mixed powder of RA in Tablettose® was prepared by mixing the ingredients for 4 hours in the cubic tumbling mixer at rotation rate of 60 rpm. This powder was packed in N° 3 white capsules by the manual filling machine. One lot of 300 capsules was prepared. The average weight of 20 capsules was 226.3 ± 2.2 mg (95% confidence interval, C.I. 95). The average RA content of ten capsules was 5.77 ± 0.19% (w/w) (C.I. 95).

Preparation of the Sample Solutions

The content of one capsule was accurately weighed and transferred to a 50 mL volumetric flask, then methanol was added to about the mark and accurately weighed. The resultant suspension, sonicated for 15 minutes under periodic shaking, was filtered through a cellulose acetate membrane into a tube closed by a PTFE lined septum screw cap.

Resolution Test Mixture

1.0 ± 0.2 mg of CRA and 2.0 ± 0.2 mg of RA were weighed into a 10 mL volumetric flask, then methanol was added to about the mark and accurately weighed; the resultant suspension was sonicated for 15 minutes under periodic shaking.

Procedure and Calculations

The preparation of all solutions was performed in a darkened room illuminated with yellow light. Although the solutions were prepared on weight basis, volumetric flasks were used as suitable containers in order to minimize solvent evaporation and to have a reference for its amount. Each solution was conserved in tubes closed by PTFE lined septa screw cap. These tubes were protected from light with Aluminium foil and stored in a refrigerator at 4 ÷ 5 °C. The containers for capsules were of amber glass.

Before use and in order to evaluate the chromatographic system, a number of system suitability tests were performed. Prior to running the system suitability checks, the column should be equilibrated for at least 30 minutes with the mobile phase flowing through the system. Acceptable results for the number of theoretical plates, tailing factor, resolution, precision, calculated using the equation of USP XXII[15], and detector linearity criteria were required before samples were analysed. The volume of each injection was not less than 80 µL.

Quantitation was accomplished using the external standard method. Every solution was injected in duplicate and the coefficient of variation (C.V.) was required < 1.5% on RA peak area basis. The RA Standard solution was interspersed with the samples if a large number of analysis were to be performed. The RA peak was always controlled for its identity by t_r and UV spectrum. The homogeneity of all chromatographic peaks was shown by the peak purity algorithm. The weight percent of RA (RA%) in the capsule content was calculated using the following equation:

$$RA\,\% = \frac{C_S}{A_S} * A_X * S * \frac{100}{W}$$

where: C_S (mg/g) is the concentration of the RA standard solution, A_S is the mean area of the RA peak from standard solution, A_X is the mean area of the RA peak from sample solution, W is the accurate weight of the content of one capsule transferred to the 50 mL volumetric flask and S is the accurate weight of methanol added to about the mark of the same volumetric flask.

The weight of each degradation product as percent of the weight of RA (DP%) was calculated using the following equation:

$$DP\,\% = Sf * \frac{A_{DP}}{A_{RA}} * 100$$

where A_{DP} and A_{RA} are respectively the peak area of each degradation product and of RA measured by integration in the same chromatogram of a sample solution, Sf is the sensitivity factor of each related substances to RA.

$$Sf = \frac{R_{RS}}{R_{RA}}$$

where R is the response factor (concentration / peak area) for the standard solutions of ERA, ORA or CRA (R_{RS}) and for RA (R_{RA}). Unknown degradation products or impurity are assigned a sensitivity factor of 1.0. The limit of detection (LOD) and the limit of quantitation (LOQ) of ERA, ORA and CRA were calculated, on peak areas, using the following equations:

$$LOD = 3 * \frac{N}{B}$$

$$LOQ = 10 * \frac{N}{B}$$

where N, the noise estimate, is the standard deviation (SD) of peak areas of five injections of each related substance solutions at the concentration of 0.1% of the RA capsule content and B is the slope of the corresponding calibration curve.

RESULTS

Selectivity and Stability Indicating Ability

The chromatograms of the solutions obtained by extraction of the capsule content of only Tablettose® maintained at room temperature and at 60°C for three weeks (Figure 1) were superimposable and did not show any interfering peaks affecting the quantitation of RA or of ORA, ERA and CRA.

The chromatogram of a RA Standard Solution spiked with ORA, ERA and CRA at the 1% level of RA showed the selectivity of this method for these related substances (Figure 2).

The stability indicating ability was shown from the typical chromatograms of one capsule maintained at shelf temperature for one year (Figure 3) and another maintained at 60°C for four months (RA assay value was 70% of the initial value) (Figure 4).

Moreover forcing degradation of RA, as drug substance, was used to demonstrate the stability indicating properties of the method. Degradation was indicated in the stressed sample by a decrease in RA assay to less than 80% of the expected value and increased levels of degradation products.

Figure 5 shows the chromatograms obtained from degrading RA under thermal conditions:

A) 25 mL of a RA Standard Solution heated by reflux (T=75°C) for 3.5 hours (residual RA 50% of the initial concentration).
B) a methanolic solution obtained from RA degraded (residual RA 50% of the expected concentration) by storage in oven at 100 °C for 24 hours and consecutively at 120 °C for other 24 hours.

Figure 6 shows the chromatograms obtained from degrading RA under other conditions:

A) a RA solution at the concentration of 0.25 mg/g in HCl/methanol 0.1 N heated by reflux for five minutes (residual RA 65% of the initial concentration).
B) a RA solution at the concentration of 0.25 mg/g in KOH/methanol 0.1 N heated by reflux for 30 minutes (residual RA 30% of the initial concentration).
C) a RA standard solution subjected, in an open container, to light (254 nm, at a distance of about 20 cm) for two hours (residual RA 50% of the initial concentration).

Linearity

Five solutions containing RA at concentration ranging from 0.12 mg/g to 0.38 mg/g (n=5, SD=0.097 mg/g and mean concentration=0.26 mg/g) were analysed. The curves of peak areas versus concentrations (Y=43.53+61427X, standard error of the estimated area S_{Yx}=81.55), heights versus concentrations (Y=-1.05+1489.3X, S_{Yx}=2.02) were linear with a coefficient of correlation r = 1.000 and the intercept values were not significantly different from zero ($p > 0.05$) in both cases.

Accuracy and Precision of the Method

The recovery studies for RA were conducted on spiked placebo formulations at three levels 50 %, 100% and 150% of the theoretical potency in three different days. The recovery study was planned in accordance with a randomized 3x3 latin square[16] (TABLE 1A) where the rows are the three different days of the assay, the columns are the run order of the samples and the treatments are the percent recoveries for the three levels. The analysis of variance (TABLE 1C) of

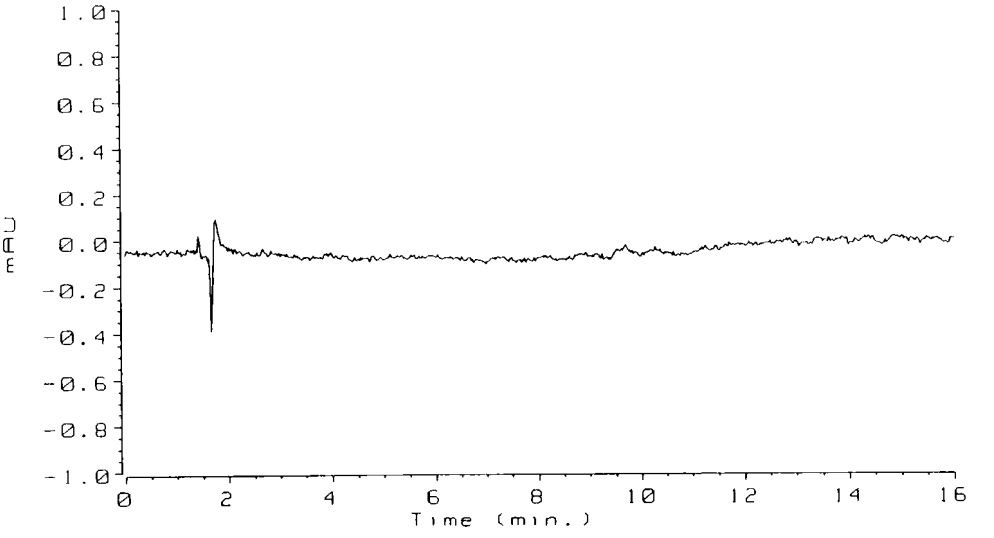

FIGURE 1

Chromatogram of a solution obtained from a capsule of only Tablettose® maintained at 60°C for three weeks.

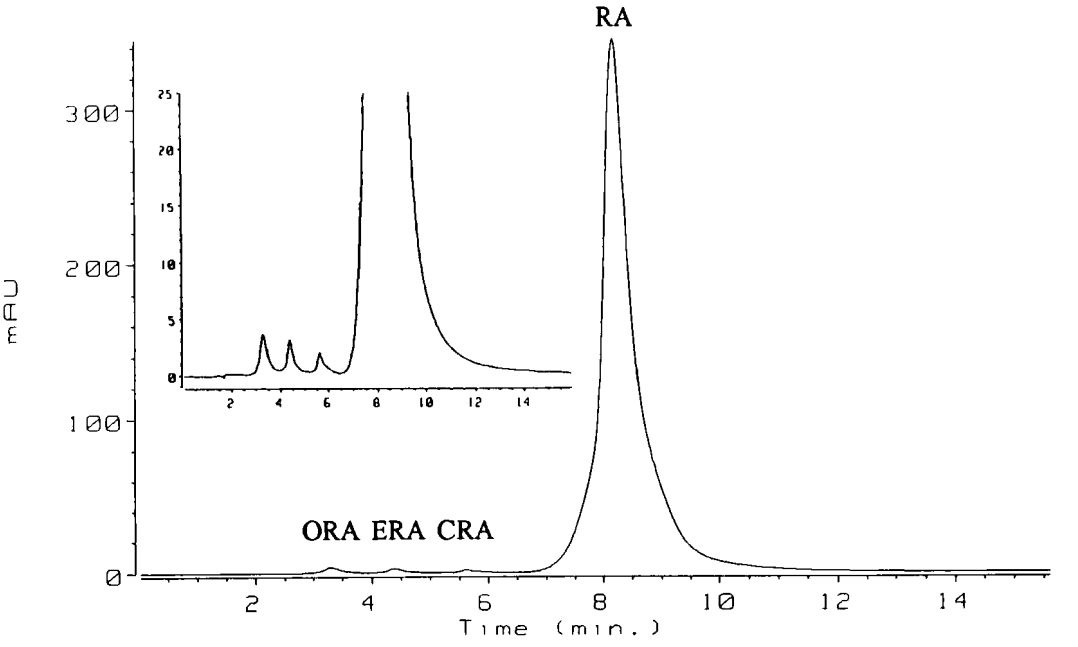

FIGURE 2

Chromatogram of a RA Standard Solution spiked with some potential degradation products at the 1% of the of RA.

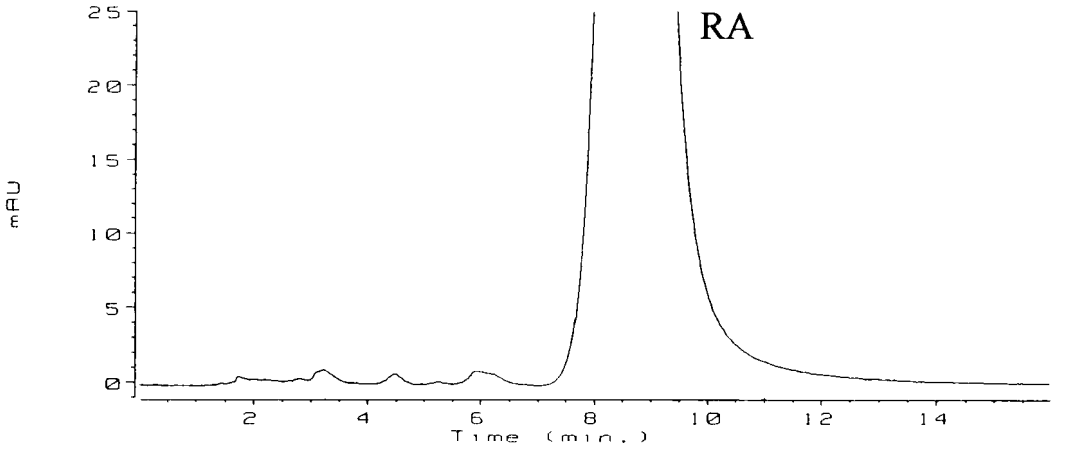

FIGURE 3

Chromatogram of a solution obtained from a capsule maintained at room temperature for one year.

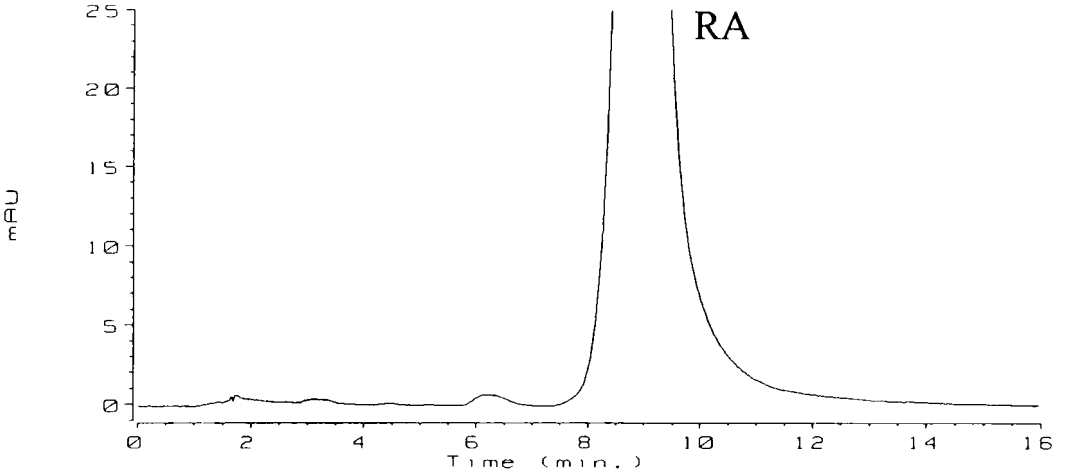

FIGURE 4

Chromatogram of a solution obtained from a capsule maintained at 60°C for four months.

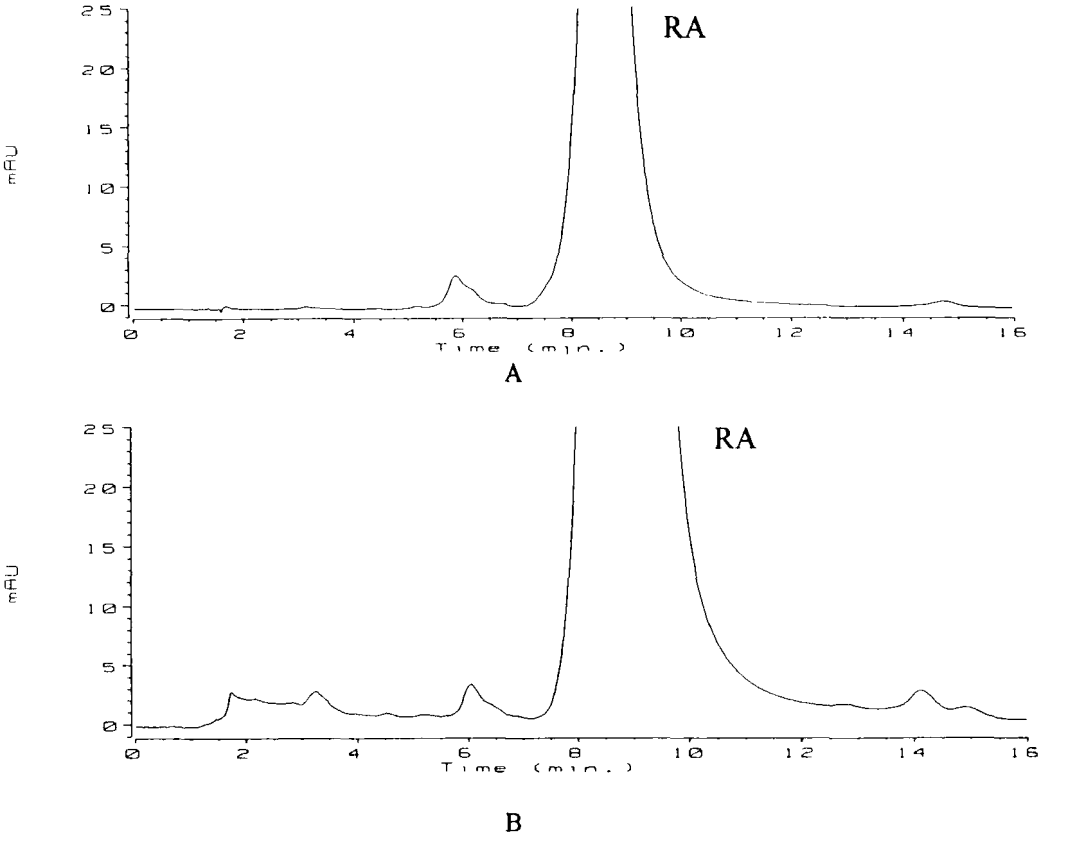

FIGURE 5

Chromatograms of: (A) 25 mL RA Standard Solution heated by reflux for 3.5 hours; (B) solution obtained from RA degraded by storage in oven at 100 °C for 24 hours and consecutively at 120 °C for other 24 hours.

the recoveries for the 3x3 latin square (TABLE 1B) does not show any significant difference ($p > 0.05$) between days, between run order and levels of the recoveries. The RA percent recovery (mean ± SD) for the nine replicates was 99.78 ± 0.36 and the C.V. was 0.4%.

Moreover the accuracy of the method was demonstrated by plotting the amount (expressed in mg) of RA found against the amount added. The linear regression analysis gave a slope not significantly different from 1 (t test, $p>0.05$), an intercept not significantly different from zero (t test, $p>0.05$) and $r = 1.000$.

The method has been applied to the stability study of the RA capsules. The assay values for two periods of the stability study of the same lot in the same storage conditions are summarized in TABLE 2 with the pertinent statistics.

Stability of the solutions

Two RA Standard Solutions and two sample solutions were stored in a refrigerator for 48 hours. At time zero and every 24 hours for 48 hours the solutions were analysed for the RA

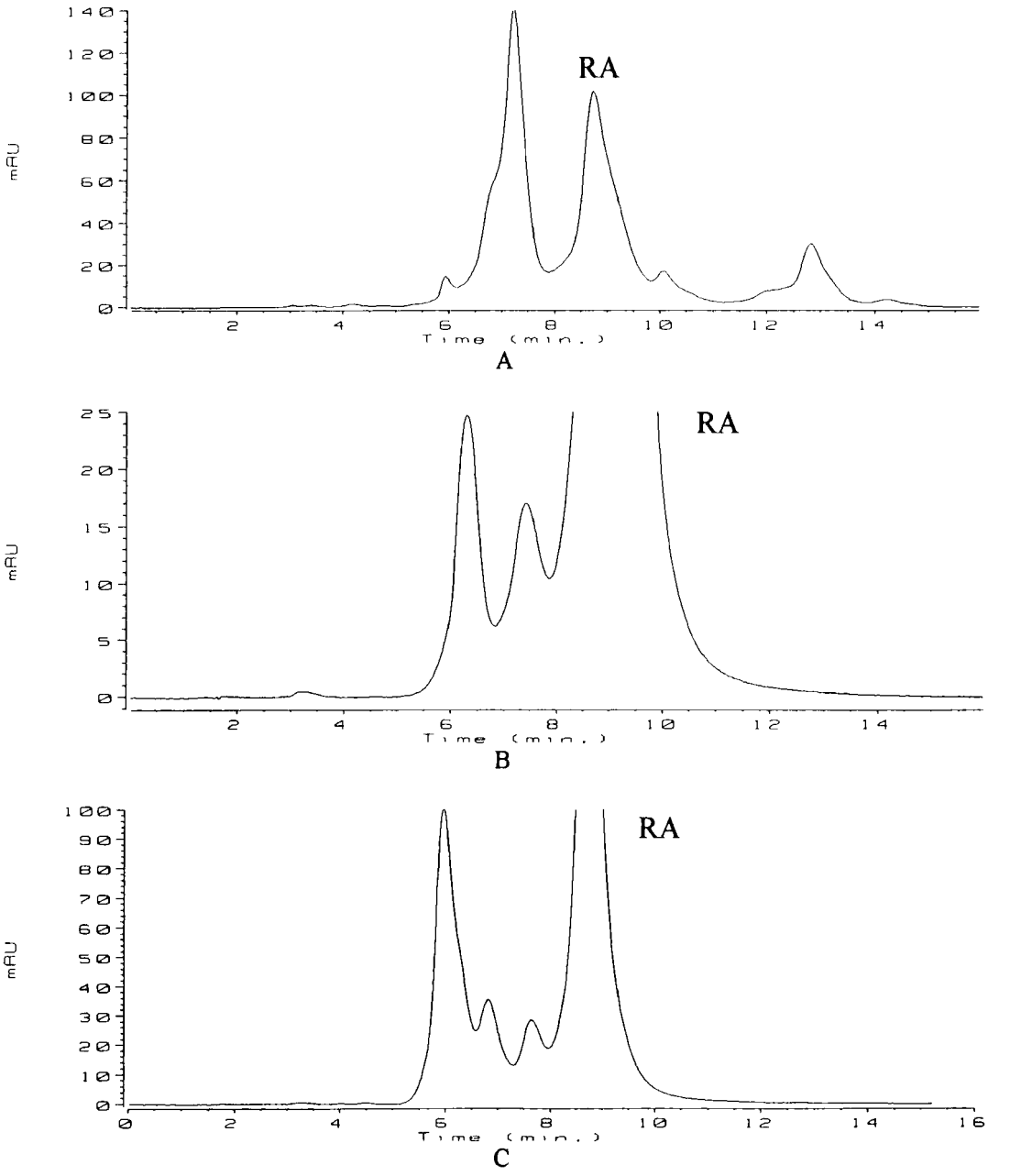

FIGURE 6

Chromatograms of: (A) RA solution at the concentration of 0.25 mg/g in HCl/methanol 0.1 N heated by reflux for five minutes; (B) RA solution at the concentration of 0.25 mg/g in KOH/methanol 0.1 N heated by reflux for 30 minutes; (C) a RA standard solution subjected to light 254 nm, for two hours (residual RA 50% of the initial concentration).

TABLE 1

Plane of the Randomized Latin Square (A); Percent Recoveries (B); Analysis of Variance for the 3x3 Latin Square (C). SS are the Sum of Squares; DF are the Degrees of Freedom; F is the Ratio Between the Mean Square MS and the Error; p is the Probability.

RUN ORDER

DAY	I	II	III
1	50	150	100
2	100	50	150
3	150	100	50

(A)

RUN ORDER

DAY	I	II	III
1	99.28	100.30	100.10
2	99.83	99.39	100.10
3	99.94	99.42	99.68

(B)

SOURCE	SS	DF	MS	F	p
RUN ORDER	0.143	2	0.071	0.823	0.548
DAY	0.069	2	0.034	0.396	0.717
LEVEL	0.660	2	0.330	3.804	0.208
ERROR	0.173	2	0.087		

(C)

TABLE 2

Assay Values (RA%) of Two Periods of the Stability Study of The Same Lot of Capsules in the Same Storage Conditions.

Capsule	ASSAY 1	ASSAY 2
1	5.85	5.52
2	5.74	5.58
3	5.67	5.42
4	5.67	5.62
5	5.47	5.24
6	5.64	5.43
7	5.73	5.41
8	5.73	5.54
9	5.83	5.61
10	5.56	5.32
mean	5.70	5.47
SD	0.11	0.13
C.V.	2.0 %	2.3 %

concentration. Five injections of each solution were runned interspersed with five injections of a RA Standard Solution freshly prepared. After 48 hours the concentrations of the tested solutions were not significantly different (*t* test, $p>0.05$) from the initial concentrations.

The stability after 48 hours in refrigerator of one RA Standard Solution and one sample solution was also confirmed using a calibration curve with $n = 5$ and the prediction of the true concentration by a 95 % two sided confidence interval [17].

Response Linearity and Recovery of ORA, ERA and CRA

The curves of peak areas versus concentrations and heights versus concentrations were linear and the intercepts were not statistically different from zero ($p>0.05$) (TABLE 3). Chromatographic and detection data are listed in TABLE 4.

The accuracy of the method for each related substance was determined at the concentration of 1% of the labelled amount of RA and the results are listed in TABLE 5.

System Suitability

The chromatographic system should be in accord with the following parameters calculated from five injections of a freshly prepared resolution test mixture: minimum number of theoretical plates in the chromatographic column of ≥ 2500 (plates/meter), calculated on the basis of the RA peak; C.V. of RA peak areas of < 1.5 %; tailing factor for the RA peak of < 1.5; resolution between RA and CRA peaks of > 2.

An injection of the resolution test mixture, diluted 1:1 with methanol, should be chromatographed and the integrated area for the RA peak should be within 48 - 52 % of that in the resolution mixture to meet the detector linearity criteria.

Ruggedness

The chromatographic system was checked evaluating alterations of the flow and of the ammonium acetate concentration on the resolution between the RA and CRA peaks of the test mixture. Changes of ± 10 % of the flow (1.125 ÷ 1.375 mL/min) or of the buffer concentration (0.9 ÷ 1.1 % p/v) had no significant effect on the separation between the two peaks.

DISCUSSION

During the method development, some observations were made that define the method as it is proposed. The use of the ultrasonic bath for a double time of that requested for a complete dissolution did not cause RA degradation. The adoption of the measure of the concentration as weight of solute on weight of solvent added was judged to be necessary because the non-negligible volume filled by undissolved lactose. On the other hand the use of an internal standard should be discouraged in the stability indicating methods because it could hide the presence of possible degradation products and mistake the assay. Moreover, working on ponderal basis makes the sample preparation more rapid than that realized on volumetric basis (a room temperature equilibrium time, after sonication, is necessary before to dilute to the mark), reducing the exposure time of RA to degrading agents like light, oxygen and temperature. Finally, this procedure involves a gain in precision[18] which is important in the stability studies of solid dosage forms.

The selectivity was demonstrated by showing that the RA peak was free of interference from the components of the dosage form. The stability indicating ability was shown because RA thermal degradation products and ORA, ERA and CRA were separated from RA.

The method was accurate (C.I. 95 of the average percent recovery was 99.5 ÷ 100.1) and the assay precision (C.V. = 0.4%) was excellent. These data indicated that the extraction procedure employed was adequate.

TABLE 3

Linearity Data for the Curves of Peak Areas Versus Concentration for ORA, ERA and CRA. RS is the related substance, n is the Number of Points; SS is the Sum of the Squares for the Concentrations; M is the Mean Concentration; S_{Yx} is the Standard Error of the Estimated Area; r is the Correlation Coefficient.

RS	CONCENTRATION RANGE (μg\g)	n	SS	M (μg\g)	EQUATION	S_{Yx}	r
ORA	0.15 ÷ 3.05	5	5.48	1.17	Y= 1.43 + 52.0X	2.87	0.999
ERA	0.14 ÷ 2.72	5	4.35	1.05	Y= - 0.81 + 55.9X	5.16	0.997
CRA	0.15 ÷ 2.97	5	5.20	1.14	Y= - 0.14 + 52.3X	1.04	1.000

TABLE 4

Chromatographic and detection data for ORA, ERA and CRA. t_R is the Mean Retention Time; n is the Number of Chromatographic Runs; λ max is the Wavelength of Maximum Absorbance; Rt_R is the Relative Retention Time to RA Peak at about 9 minutes.

Related substance	λ max (nm)	t_R(min) (C.V., n)	Rt_R	LOD (μg\g)	LOQ (μg\g)	Sf
ORA	356	3.3 (1.9%, 27)	0.6	0.06	0.20	0.93
ERA	327	4.6 (2.6%, 28)	0.5	0.03	0.11	0.89
CRA	346	6.3 (0.9%, 26)	0.3	0.13	0.43	0.88

TABLE 5
Percent Recoveries Data for the Related Substances.

sample	ORA	ERA	CRA
1	100.0	98.4	100.9
2	98.5	98.1	98.6
3	99.4	98.2	102.6
4	98.5	98.1	101.8
5	99.3	99.6	101.2
MEAN	99.1	98.5	101.0
SD	0.6	0.6	1.5

The effects of the factors considered for the validation procedure were additive and without interaction, this made possible the use of the latin square like experimental design. The latin square produces a substantial reduction in error over the randomized blocks design. The results of the validation showed that the method was not affected by the day of assay, the RA amount considered and the run order of analysis. For the latest reason the analysis of three capsules prepared at the same time is practicable. The sample preparation is rapid and the resultant solutions can be stored in refrigerator for 48 hours without alteration.

The assays of RA and of the degradation products were performed by the same chromatographic run.

From the data in TABLE 4 it draws that the method is able to detect the presence of ORA, ERA and CRA respectively at 0.02%, 0.01%, 0.05% of the RA labelled amount (10 mg) and to quantitate them at level of 0.1%, 0.04% and 0.2%. The accuracy and the precision of the method for these related substances were good. To enhance the sensitivity for degradation products the DAD can be set at λ max of every analyte (TABLE 4).

CONCLUSIONS

The analytical procedure described represents a selective, linear, precise, accurate and stability indicating method for the determination of RA in hard capsules containing lactose as diluent.

This procedure has been successfully employed for more than one year and a half in a stability study and assay of this dosage form, and may be applied to the stability studies of RA as bulk drug or for its assay and determination of its chromatographic purity.

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