Analytical Methods

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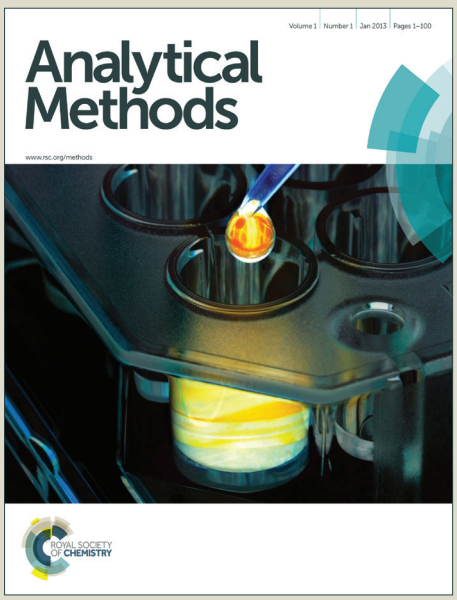

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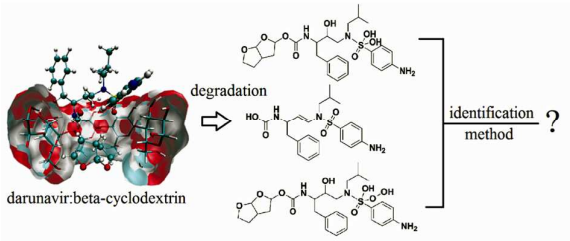

►A method was developed to identify degradation products of the complex darunavir:β-cyclodextrin. ►This type of research affects the whole pharmaceutical industry chain, the patient and the healthcare system.

Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

Stability indicating thin layer chromatographic method for determination of darunavir in complex darunavir:β-cyclodextrin in presence of its degradation products

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Darunavir (DRV), a protease inhibitor used in the treatment of HIV infection, presents low water solubility and poor bioavailability. Therefore, the complexation of DRV with β-cyclodextrin (β-CD) was performed. This drug is not described in official compendiums. Simple, selective and inexpensive stability-indicating thin-layer chromatographic (TLC) method for the determinations of DRV in complex DRV:β-CD and its degradation products was developed. The TLC method employs aluminum plates precoated with silica gel 60_{F-254} as the stationary phase and purified water and methanol, 70:30 (v/v) adjusted to pH 2.4 with glacial acetic acid as solvent system to provide spots for DRV (R_f=0.66) and its degradation products acidic (R_f=0.73 and 0.76), basic (R_f=0.53) and oxidative (R_f=0.71, 0,75 and 0.84). The chromatogram was visualized in chamber UVA at 365 nm. HPLC analysis was performed on a Waters HPLC system, Phenomenex CN Luna (250 x 4.6 mm) column and mobile phase consisting of water + 0.1 % glacial acetic acid and acetonitrile + 0.1 % glacial acetic acid in the ratio 60:40 (v/v) at a flow rate of 1.0 mL min^{-1} and 268 nm for the separation of DRV (t_R=7.3) and its degradation products acidic (t_R=5.1 and 6.7 min), basic (t_R=7.8 min) and oxidative (t_R=5.1, 5.5 and 6.7 min). DRV:β-CD was subjected to acid and alkali hydrolysis and oxidation and analyzed by the proposed methods in presence of its degradation products, which were identified by LC-MS. As the methods could separate the DRV from their degradation products, these techniques can be employed as indicative stability methods and can be effectively applied in quality control of DRV complexed to β-CD.

A Introduction

Darunavir (DRV), a protease inhibitor used in the treatment of HIV infection, is a new generation of synthetic non-peptidic protease inhibitor,[1,2] a pillar of therapy cocktail for patients with the virus.

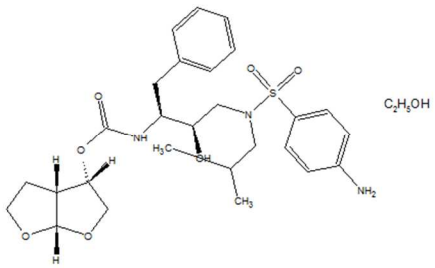


Fig. 1 Chemical structure of DRV (CAS 206361-99-1).

DRV presents low water solubility and poor bioavailability,[2] therefore the complexation of DRV to β-cyclodextrin was performed. The β-CD is an excipient and it has been used in the development of pharmaceuticals, particularly because of complexing properties, which provide increased solubility and consequent increase in the rate of dissolution of poorly soluble drugs.[3].

DRV is not described in the USP 35,[4] Brazilian,[5] British[6] and Portuguese[7] Pharmacopoeias and only one paper was found in the literature which describe the technique of thin layer chromatography (TLC) for this drug in pharmaceutical form tablets[8] and nothing has been found about techniques that identify and quantify the DRV in complex DRV:β-CD.

Among the modern methods of analysis, high performance liquid chromatography (HPLC) occupies a prominent place because of its ease in effecting the separation, identification and quantification of chemical species.

The TLC is an important tool to identify compounds, being easy to perform, versatile, inexpensive and enables to check the presence of impurities and degradation products.[9]

The HPLC equipment is expensive compared to the cost required to perform the technique of TLC. Thus, if exists the possibility for the identification and investigation of the degradation products in the TLC, it is valid. The developments of cheaper, functional and reliable methods, today more and more,

are preferred in the pharmaceutical industry to reduce costs and optimize equipment.

Stability of pharmaceuticals is extremely important for the success of the therapeutic effect. Currently, the development of appropriate stability indicating methods has increased enormously.[10]

Thus, this work reports for the first time a stability indicating TLC method to identify the DRV complexed to β-CD and its degradation products. These results were compared with the results obtained by HPLC method and its degradation products were identified by LC-MS.

B Experimental

Instrumentation and reagents

Instrumentation. The migration chromatography was performed on silica gel plates 60_{F-254} (20 x 20 cm) thickness of 0.25 mm (Merck). The spots were visualized using UVA ultraviolet lamp at 365 nm.

HPLC analysis was performed on a Waters HPLC system equipped with Waters 1525 binary gradient chromatography pump, Rheodyne Breeze 7725i manual injector and Waters 2487 UV-Vis detector, LC-MS analysis was performed on Shimazdu® HPLC system connected to AmaZon SL Bruker® ion trap mass spectrometer operating in positive ion electrospray ionization mode, Phenomenex CN Luna (250 x 4.6 mm) column, analytical balance model 410 Kern (Kern), ultrasonic bath Ultrasonic Cleaner (Unique), water purification system (Millipore) and membranes of polytetrafluoroethylene (PTFE) hydrophilic with pore 0.45 µm and diameter 47.0 mm (Millipore).

Chemicals and reagents. The chemical used was complex DRV:β-CD. The samples were prepared in ethyl alcohol, hydrochloric acid (HCl) $1x10^{-2}M$, sodium hydroxide (NaOH) $1x10^{-2}M$ and hydrogen peroxide (H_2O_2) 3%. The acidic, basic and oxidative solutions were subjected to bath at 80ºC for 8 hours and then analyzed by TLC, without being treated or neutralized in the case of acidic and basic solutions.

The eluent system of TLC was composed of a mixture of purified water, methanol (Synth) and glacial acetic acid (Qhemis), both of analytical grade. The system of mobile phase used was purified water and methanol, 70:30 (v/v) adjusted to pH 2.4 with glacial acetic acid.

In the HPLC and LC-MS, the chemical used were acetonitrile (J.T.Baker), glacial acetic acid (Synth) HPLC grade and deionised Milli Q water (Millipore). Mobile phase was prepared by mixing water + 0.1 % glacial acetic acid and acetonitrile + 0.1 % glacial acetic acid in the ratio 60:40 (v/v) filtered through 0.45 µm membrane filter.

Pharmaceutical preparations. A pool of complex DRV:β-CD was used. A quantity of powder equivalent to 100 mg of DRV weighed accurately into a 100 mL calibrated flask; 50 mL of diluent solution was added and sonicated for 30 min to complete dissolution; then the mixture was diluted to the mark with the diluent. A portion of the resulting mixture was withdrawn and filtered through a 0.45 µm filter to ensure the absence of particulate matter.

Methods

The silica gel plates were activated at 150ºC for 1 hour and then 5 µL of the samples prepared in ethyl alcohol, HCl $1x10^{-2}M$, NaOH $1x10^{-2}M$ and H_2O_2 3% were applied in the plates. Then, proceeded to saturation glass chamber with a system of mobile phase consisting of purified water and methanol, 70:30 (v/v) adjusted to pH 2.4 with glacial acetic acid. After migration of the mobile phase, the plate was removed from the glass chamber, allowing the solvent to evaporate. The plates were developed in a UVA chamber at 365 nm for evaluation of the spots and determination of the R_{fs} of DRV and its degradation products. The R_f value was obtained by dividing the distance of migration of the drug and the distance traveled by the eluent.[8]

HPLC analysis was carried out at ambient temperature (25°C) on a Phenomenex CN Luna (250 × 4.6 mm) column. The mobile phase was a mixture of water + 0.1 % glacial acetic acid and acetonitrile + 0.1 % glacial acetic acid (60: 40, v/v). Flow rate was 1.0 mL min^{-1} and the detector wavelength was set at 268 nm, with injection volume at 20 µL. LC-MS analysis were performed on AmaZon SL provided with ESI ion source and ion trap mass analyser. Analyses were carried out using nitrogen as nebulizing (60 psi) and drying gas (10 L min^{-1}, 320ºC). The capillary high voltage was set to 3500 V

C Results

Values of R_f obtained after chromatographic migration were 0.66 for DRV, 0.73 and 0.76 for the products of degradation in acid media, 0.53 for the products of degradation in basic media and 0.71, 0.75 and 0.84 for the products of degradation in oxidative media.

The chromatographic profiles of the samples after visualization in chamber UVA at 365 nm can be observed in Figure 2.

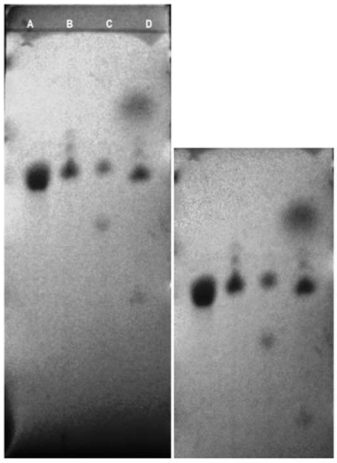


Fig. 2 TLC of DRV:β-CD under stress in concentration of 1 mg mL^{-1} using purified water and methanol, 70:30 (v/v) adjusted to pH 2.4 with glacial acetic acid as mobile phase. **a)** (A) DRV R_f=0.66, (B) DRV R_f=0.67 and acid degradation products with R_{fs}=0.73 and 0.76, (C) basic degradation product with R_f=0.53 and DRV R_f=0.67, (D), H_2O_2 R_f=0.37, DRV R_f=0.67 and oxidative degradation products with R_{fs}=0.71, 0.75 and 0.84; **b)** zoom of observed stains.

Hydrogen peroxide can be visualized both on the TLC plate, R_f=0.37, as in the HPLC chromatogram, t_R= 3.2 min.

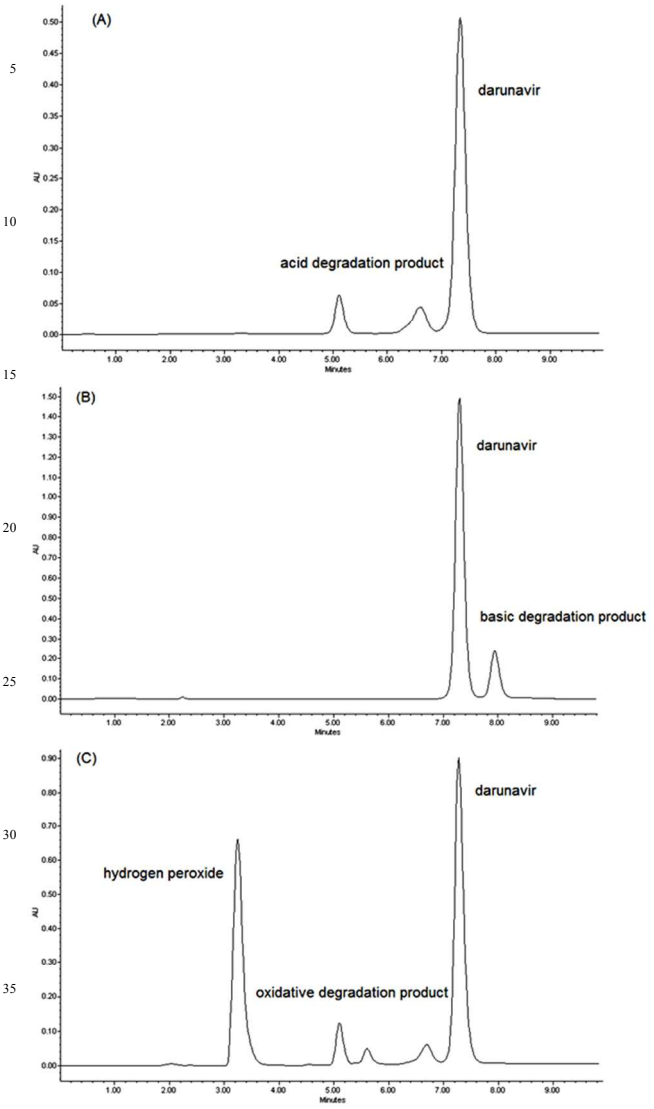


Fig. 3 Degradation products acidic, basic, and oxidative of complex DRV:β-CD in concentration of 1 mg mL^{-1} obtained by HPLC using water + 0.1 % glacial acetic acid and acetonitrile + 0.1 % glacial acetic acid in the ratio 60:40 (v/v) as mobile phase. (A) acid degradation products with t_R=5.1 and 6.7 min, (B) basic degradation product with t_R=7.8 min, (C) H_2O_2 t_R=3.2 min and oxidative degradation products with t_R=5.1, 5.5 and 6.7 min.

Samples of acidic, alkaline and oxidative degradation were submitted to analysis of LC-MS to identify the degradations products generated (Table 1). Acidic degradation generated 2 products, the first one showed *m/z* 588 $[M+Na]^+$ and the other 616 $[M+Na]^+$. 2 of the 3 oxidative degradation products showed the same retention times of acidic degradation products and the same *m/z* 588 $[M+Na]^+$ and the other 616 $[M+Na]^+$. The third presented *m/z* 604 $[M+Na]^+$. Basic degradation generated 1 product with *m/z* 416 $[M-H]^-$ (observed in MS spectra in negative mode).

The R_f values from the TLC technique for the DRV and its degradation products were analyzed to show that their values are statistically different. The results were analyzed by Student's t test (Table 2) and confirmed the difference between the spots.

Table 2 Statistical comparison the results of the spots obtained in the TLC technique for DRV and its degradation products

Products	R_{fs}	Student´s t test	
		F_{cal}	F_{tab} (0.05)
DRV	0.66		
Acid degradation	0.73	7.35*	2.78
	0.76	11.02*	2.78
Basic degradation	0.53	17.15*	2.78
Oxidative degradation	0.71	4.90*	2.78
	0.75	9.80*	2.78
	0.84	20.82*	2.78

*Significant for p<5%

D Discussion

Today HPLC technique is preferred in routine analysis in laboratories for identification and investigation of degradation products of drugs and pharmaceuticals. However, if there is another cheaper and reliable method to demonstrate the same results it should be considered to replace the HPLC or to reduce the generation of waste.[11,12]

The TLC is a flexible technique because it allows the use of various eluent systems and revealing agents. It is an important tool to identify compounds, being easy to perform, versatile and low cost.[9] Another application of the TLC refers to the possibility to check the presence of impurities and degradation products in the sample.[13]

Various solvent systems were tested and one of them was elected the best for both identification of DRV as to detect possible contaminants through the appearance of other spots along the distance. Thus, the mobile phase containing purified water and methanol, 70:30 (v/v) adjusted to pH 2.4 with glacial acetic acid was chosen. Visualization of the spots was easily observed in UVA chamber.

In the test of TLC proposed were observed the same degradation products obtained by HPLC method previously performed (Figure 3). In both methods were found two degradation products derived from acidic condition (t_R=5.1 e 6.7 min), one of the basic condition (t_R=7.8 min) and three of the oxidative condition (t_R=5.1, 5.5, 6.7 min). Thus, all products from acid, basic and oxidative degradation of complex DRV: β-CD observed in method HPLC were reproduced in TLC method developed.

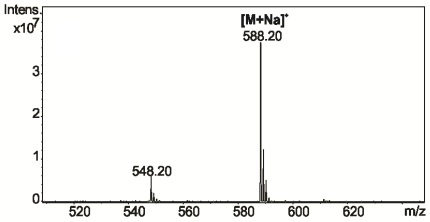
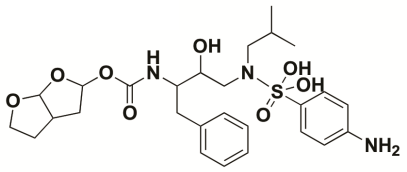
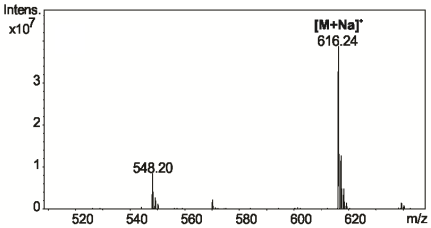
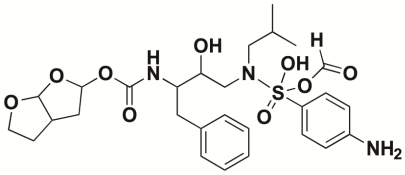
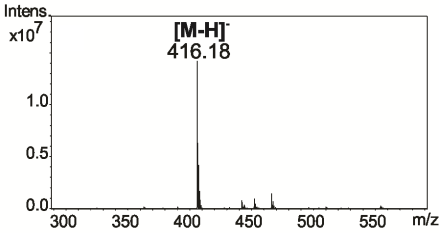
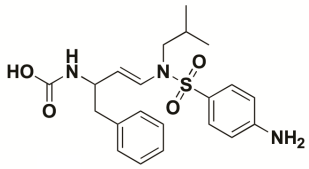
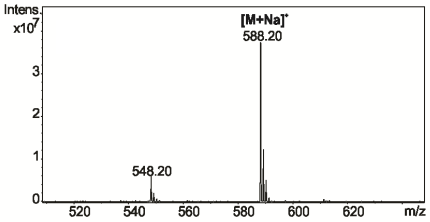
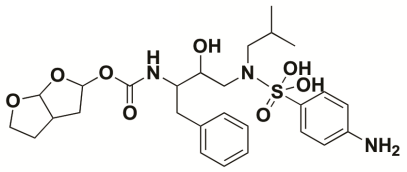
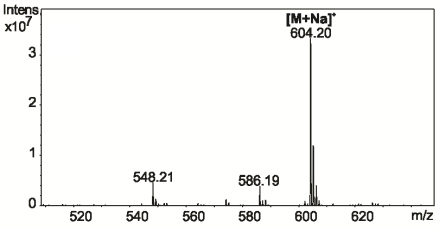
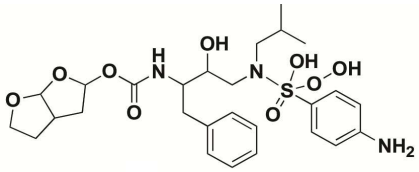
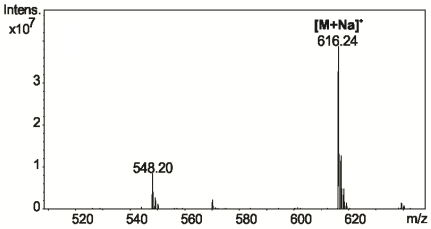
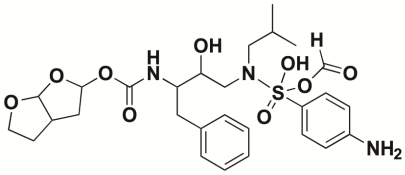
Furthermore, analysis of LC-MS was done. In accord to results obtained, it was possible suggested the degradations products by cationized molecule in positive mode and deprotonated molecule in negative mode (Table 1). By analysis of HPLC and TLC we can see the same retention time for two degradations products from acid and oxidative degradation. In this way, it was observed two compounds with the same molecular mass for this degradations conditions and only one compound different in oxidative degradation. The basic

degradation occurs by different mechanism and the analysis was done in negative mode, since the ionization is more favorable.

As in the HPLC system the column used was reverse phase, the non polar product were retained more time on the column, generating an t_R higher. In TLC more polar components eluted more easily with the mobile phase generating R_{fs} larger and leaving faster retained the non polar components, with R_{fs} smaller. For this reason, the appearances of the DRV and its products occur inversely in HPLC and TLC.

The R_{fs} of spots of the degradation products were analyzed concerning their differences by Student's t test and all showed R_f statistically different to the R_f of DRV, at a significance level of 5%. This shows that retention of the product compared to DRV is different, which facilitates the observation of the spots on the plate.

Table 1 LC-MS spectra and proposed structures of degradation products of the complex DRV:β-CD

Degradation product	Mass spectrum	Proposed structure
Acid t_R = 5.1 min		 MW=565
Acid t_R = 6.7 min		 MW=593
Basic t_R = 7.8 min		 MW=417
Oxidative t_R = 5.1 min		 MW=565
Oxidative t_R = 5.5 min		 MW=581
Oxidative t_R = 6.7 min		 MW=593

Using the technique of TLC developed, it was possible to view all degradation products observed in the HPLC method. This result was achieved faster, cheaper and generate less

residues. For this it was not necessary equipment HPLC, column, software and time to prepare and wash the column.

The investigation of alternative methods such as this should be valued, as well as to think about the socioeconomic impact of analytical decisions. If the cost of acquisition, maintenance, production, analysis and conditioning of drugs and pharmaceuticals is high, consequently the price of this product in the market will be higher and it cannot be accessible to the patient. Treatment failure not only affects the quality of life of patients, but also contributes significantly to the economic burden of the health system.

E Conclusion

The TLC developed was suitable for the identification of DRV in complex DRV:β-CD and its acid, basic and oxidative degradation products, being appropriate for use in routine Quality Control of this drug.

F Acknowledgments

The authors acknowledge CNPq (Brasília, Brazil), FAPESP (São Paulo, Brasil), CAPES (São Paulo, Brasil) and PADC/FCF/UNESP (Araraquara, Brazil).

This paper is dedicated to School of Pharmaceutical Sciences on the occasion of its 90th anniversary.

G Notes and references

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1 E. V. Gyseghema, S. Stokbroekx, H. N. Armas, J. Dickensb, M. Vanstockem, L. Baert, J. Rosier, L. Schueller, G. V. Mooter, *Eur. J. Pharm. Sci.,* 2009, **38**, 489.

2 Sosnik, D. A. Chiappetta, A. M. Carcaboso, *J. Controlled Release,* 2009, **138**, 2.

3 M. A. Lyra, L. D. S. Alves, D. A. F. Fontes, J. L. Soares-Sobrinho, P. J. Rolin-Neto, *Rev. Ciênc. Farm. Básica Apl.,* 2010, **31**, 117.

4 *The United States Pharmacopeia*, USP Convention, 35th edn., 2012.

5 *Brazilian Pharmacopeia*, Brasília, Brazil, 5th edn, 2010.

6 *British Pharmacopoeia*, The Stationary Office, London, 2010.

7 *Portuguese Pharmacopeia*, Infarmed, Lisboa, 8th edn, 2005.

8 C. Kogawa, H. R. N. Salgado, *Rev. Ciênc. Farm. Básica Apl.*, 2013, **34**, 207.

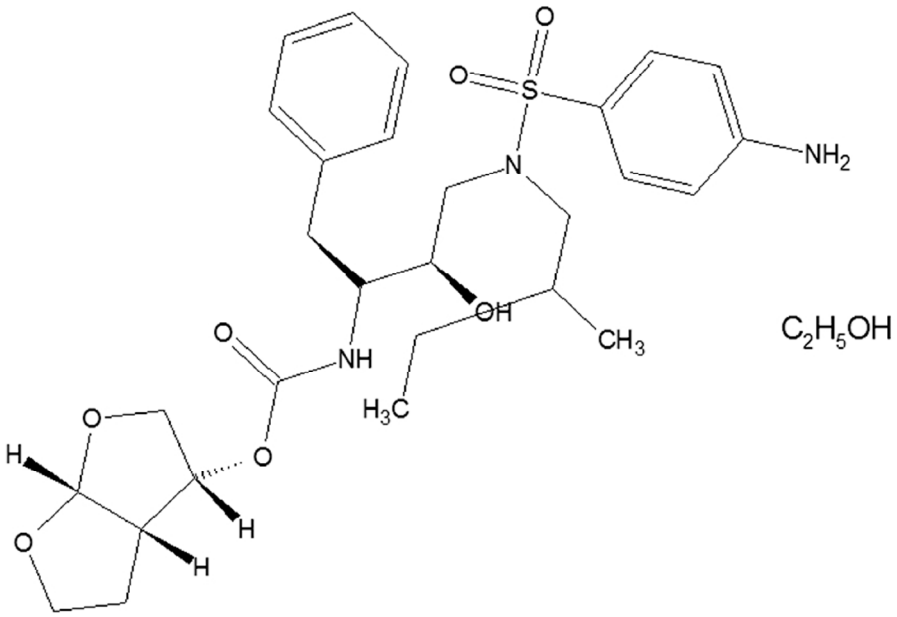
9 S. R. Dhaneshwar, V. K. Bhusari, M. V. Mahadik, *J. AOAC Int.* 2009, **92**, 387.

10 H. Salem, A. A. Moustafa, M. Hegazy, O. Ali, *Pharm Anal Acta,* 2013, **4**, 1.

11 E. G. Tótoli, H. R. N. Salgado, *Anal. Methods*, 2013, **5**, 5923.

12 R. A. Sversut, M. S. do Amaral, A. C. M. Baroni, P. O. Rodrigues, A. M. Rosa, M. C. G. Gerlin, A. K. Singh, N. M. Kassab, *Anal. Methods*, 2014, **in press**.

13 D. G. Watson, *Pharmaceutical analysis: a textbook for pharmacy students and pharmaceutical chemists,* Edinburgh, Elsevier Churchill Livingstone, 2005.



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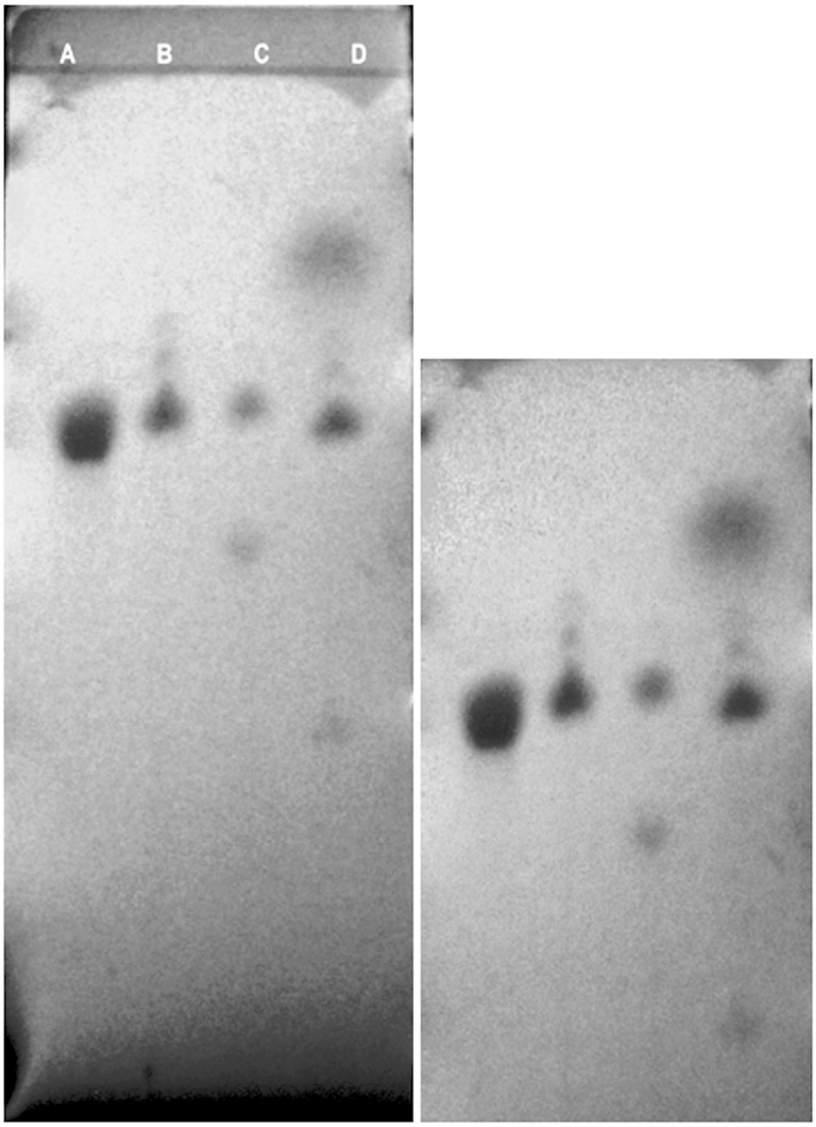

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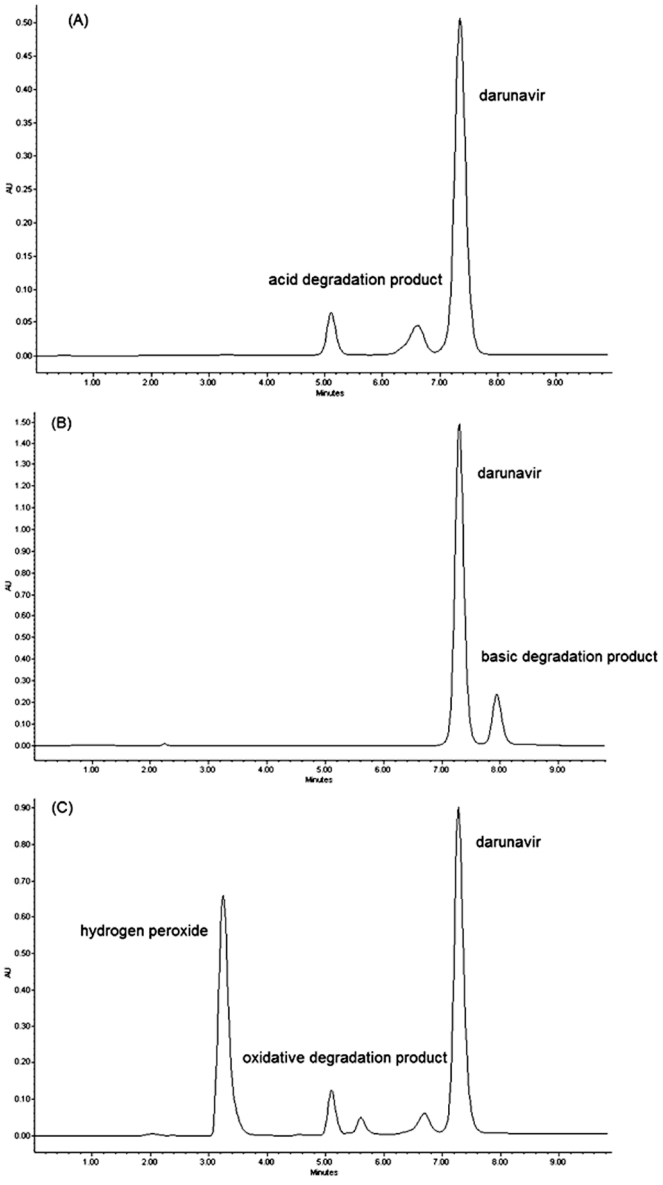

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