Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 678–685
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development of a gas chromatography method for the determination of isotretinoin and its degradation products in pharmaceuticals

Eliana Martins Lima [a,*], Danielle G. Almeida Diniz [a], Nelson R. Antoniosi-Filho [b]

[a] *Lab. Tecnologia Farmaceutica, Faculdade de Farmacia, Universidade Federal de Goias, Av. Universitaria, 74605-220 Goiania, GO, Brazil*
[b] *Instituto de Quimica, Universidade Federal de Goias, Campus 2, Goiania, GO, Brazil*

Received 18 February 2004; received in revised form 27 January 2005; accepted 11 February 2005
Available online 31 May 2005

Abstract

This paper describes the development of a gas chromatography (GC) method used for the assay of isotretinoin in its isolated form and in pharmaceutical formulations. Isotretinoin soft and hard gelatin capsules were prepared with various excipients. The performance of the proposed gas chromatography method was compared to that of traditional high performance liquid chromatography (HPLC) systems for this substance, and the GC parameters were established based on several preliminary tests, including thermal analysis of isotretinoin. Results showed that gas chromatography-flame ionization detector (GC-FID) exhibited a separation efficiency superior to that of HPLC, particularly for separating isotretinoin degradation products. This method was proven to be effectively applicable to stability evaluation assays of isotretinoin and isotretinoin based pharmaceuticals.

Keywords: Isotretinoin; Gas chromatography; Assay; Stability

1. Introduction

Isotretinoin or 13-*cis*-retinoic acid is a member of the large group of Vitamin A related compounds. Due to its effect on regulating cell differentiation it has been used for the treatment of cystic and nodular acne and also as an inhibitor of neoplasic cells proliferation [1–8].

Isotretinoin is a highly unstable compound, and its shelf life depends mostly on the storage conditions, particularly atmospheric temperature, oxygen and light [9].

High performance liquid chromatography (HPLC) has been the analytical method of choice for the analysis of retinoids and gradually became the preferred method for separation and quantification of retinoids in biological samples and pharmaceutical products. A large number of natural and synthetic retinoids with different polarities is available. The development of an analytical method capable of separating all the retinoids in only one chromatographic analysis has been proven to be a very difficult task. Several procedures have been tested for the quantitative determination of natural and synthetic retinoids in biological samples and in pharmaceutical dosage forms. The majority of these methods involve reverse-phase HPLC and require an extremely sensitive system capable of separating the different geometrical isomers and degradation products of retinoids (Table 1) [10,11].

Due to retinoids' photoinstability, thermal instability and high oxidative rate, their quantitative determination in pharmaceutical dosage forms is particularly important for the quality control of the final product and stability indicating assays [12]. Lehman and Malany [13] used gas chromatography–mass spectrometry (GC–MS) to assess elution peaks for isotretinoin and tretinoin obtained by HPLC. Gas chromatography has also been used as an additional method for comparing and identifying retinoids and their reference compounds [14].

* Corresponding author. Tel.: +11 55 62 209 6044; fax: +11 55 62 209 6037.
E-mail address: emlima@farmacia.ufg.br (E.M. Lima).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.02.015

Table 1
Chromatographic techniques used for qualitative and quantitative detection and determination of retinoids

Sample origin	Pre-column	Stationary phase	Mobile phase (MP)	Detector	Reference
Plasma	PEEK® 20 × 4. 0 mm, Pelliguard C8	Bondapak® C1837–53 μm, Supplex 250 × 4 mm.	MP1a: tetrafluoracetic acid 0.05%/acetonitrile (85:15) MP1b: 100% methanol MP2: acetonitrile/1-butanol/methanol/ammonium acetate 2%/acetic acid glacial (69:2:10:16:3)	UV (λ = 360 nm)	[10]
Pharmaceuticals	Not used	Phenomenex Prodigy® 5 ODS_3® (250 × 3.2 mm)	Acetonitrile/ethanol/acetic acid glacial 1% (68:8:24)	Fluorescent light (λ_{em} = 520 nm, λ_{ex} = 350 nm).	[12]
Human skin		HPLC 1: C18 Resolve® (Watters) 5 mm × 15 cm at 25 °C	MP1: methanol/acetonitrile/buffer (2.5% Ammonium acetate e 0.1 M maleic acid) (50:25:25)	UV λ = 350 nm	[13]
		HPLC2: C18 Ultraspher® (Altex) 4.6 mm × 25 cm at 40 °C	MP2: methanol/H_2O/acetic acid glacial (95:4.5:0.5)	UV λ = 350–360 nm UV scan 210–500 nm	
		GC: DB-5-30W® (0.2 mm × 30 m) 3 min at 180 °C to 220 °C	He	SIM m/e 314	
Plasma	Not used	System I: Spherisorb® ODSII 3 μm(120 × 4 mm)	MP1a: 60 mM ammonium acetate (pH 5.75) acetic acid/methanol(1:1)	UV λ = 340 nm	[14]
		System II: Nucleosil 5®C18 (120 × 4 mm)	MP2b: methanol		
		System III: Hypersil ODS® 5 μm (300 × 4 mm)	MP3a: 10 mM TBAHS/20 mM HEPES® (pH 7)/methanol (3.5:6.5) ou FMIIb 10 mM TBAHS®/20 mM HEPES® (pH 7)/methanol (1:4) a 35 °C MP3b: 60 mM ammonium acetate (pH 5.75)/methanol (1:4) ou FMIIIb 60 mM ammonium acetate (pH 5.75)/methanol (1:9) a 30 °C		
Plasma		GC: DB®-5-30W (0.2 mm × 20 m)	He	Mass detector	[14]
Drug	Used (not specified)	Hypersil® ODS C18 (4.6 × 15 cm)	Acetonitrile/ammonium acetate 1% (95:5)	UV λ = 280 nm	[15]
Solution	Used (not specified)	C18 HypersilODS® (4.6 mm × 15 cm)	Acetonitrile/ammonium acetate 1% (95:5)	UV (λ = 360,280 e 254 nm)	[16]
Pharmaceuticals	Not used	Phenomenex Luna® 3 μm C18 (180 × 4.6 mm).	MP1a: methanol/10 mM ammonium acetate (75:25)	UV-diode array (λ = 350 nm)	[18]
		Phenomenex Prodigy® 5ODS_3 (250 × 3.2 mm).	MP1b: methanol/THF (84:16) MP2: acetonitrile/ethanol/THF (73:25:2)	Fluorescent light (λ_{em} = 520 nm, λ_{ex} = 350 nm).	
Plasma	Not used	Nova-Pak® C18 (3.9 ×150 mm)e Waters C18 (3.9 × 75 mm)	MPa:acetonitrile MPb:ammonium acetate 0.1 mol/l pH 5 acetic acid	Mass detector	[19]
Plasma	Lichrocart® 250 × 4 mm	Lichrospher® SI 60 (250 × 4 mm)	Hexane/diclhoromethane/dioxano 1% acetic acid glacial (78:18:4)	UV λ = 360 nm	[20]
Hard gelatin capsule	Lichrosorb® RP-18 (5 μm)	Lichrospher®100 RP-18 (5 μm)	Acetonitrile/ammonium acetate 1% (90:10)	UV-diode array λ = 340 nm bandwidth 4 nm/reference λ = 500 nm bandwidth 80 nm	[21]
Plasma	Used (not specified)	Lichrospher® 100 RP-18 (5 μm), Superspher 100 RP-18	MP1a: ammonium acetate10%/H_2O/ethanol/acetic acid glacial (1:7:2:0.1); MP1b: ammonium acetate 10%/H_2O/ethanol/acetic acid glacial (1:9:0.4:0.2); MP2a: ammonium acetate 10%/H_2O/acetic acid/acetonitrile (6:30:1:60); MP2b:ammonium acetate 10%/H_2O/acetic acid/acetonitrile (5:20:20:950); MP2c:H_2O/acetic acid/acetonitrile (5:5:990); MP3a: ethanol/H_2O e FM3b: ethanol	UV λ = 230 nm	[22]
Plasma	Not used	Nova Pak®C18	MPa: methanol/acetonitrile/THF 5% (65:35) MPb: acetic acid glacial 2%	UV λ = 350 nm	[23]

Table 1 (*Continued*)

Sample origin	Pre-column	Stationary phase	Mobile phase (MP)	Detector	Reference
Drug	Used (not specified)	Hypersil® ODS C18 (4.6 × 15 cm)	Acetonitrile/ammonium acetate 1% (95:5)	UV λ = 280 nm	[15]
Drug	Not used	ZorbaxRx-Sil® (25 cm × 4.6 mm) 5 μm	Heptane/THF/acetic acid glacial (96.5:3.5:0.015)	UV λ = 365 EI mass 77 eV, 200 m/z	[24]
Plasma	Not used	Inertsil SILICA® 100–105 (4.6 mm × 25 cm)	MPa: n-hexane/2-propanol/acetic acid glacial (1000:4.3:0.675) MPb: *n*-hexane/2-propanol/acetic acid glacial (1000:17.5:0.675)	UV λ = 350	[25]
Plasma	Not used	C185 μm (4.6 mm × 25 cm) da Alltech®	Methanol/0.1% ethanol e triethylamine (85:15)	UV λ = 245 nm	[26]
Pharmaceuticals	Lichrocart® (250 × 4 mm)	Lichrospher® 100 RP-185 μm	MPa: methanol/H_2O/acetic acid (75:12.5:1) MPb acetonitrile/H_2O/acetic acid (80:20:1)	UV λ = 320–350 nm	[27]
Pharmaceuticals	Not used	Spherisorb ODS® 2 (250 × 4.6 mm)	Methanol/acetonitrile/acetic acid 0.05%(42.5:32.5:25)	UV λ = 360 nm	[28]

Stability studies of isotretinoin using calorimetric, mass spectrometry, HPLC and crystallographic methods have demonstrated severe physical and chemical modification in the characteristics of the product after exposure to oxygen, light, humidity and high temperatures [15–17].

In this study, an analytical method using gas chromatography for the determination of isotretinoin in pharmaceutical dosage forms was developed and subsequently used to evaluate isotretinoin stability.

2. Experimental

2.1. Materials

Isotretinoin samples were obtained from the following products: raw material (RM) for pharmaceuticals, isotretinoin commercial soft gelatin capsules (reference and generic brands), isotretinoin hard gelatin capsules from a compounding pharmacy and United States Pharmacopeia reference standard number 353500 (Rockville, MD, USA).

Hexane, ethyl acetate and dicloromethane (HPLC grade) were purchased from Merck Co. (Darmstadt, Germany). All other chemicals were analytical grade or higher. Oxygen 2.8, Nitrogen 5.0, Hydrogen 4.5 and Synthetic air 4.7 (FID) were purchased from White Martins (Sao Paulo, Brazil).

2.2. Chromatographic system

An Agilent 6890 gas chromatography system (Palo Alto, USA) equipped with a flame ionization detector and a Shimadzu LC10Avp (Kyoto, Japan) HPLC system were used. Columns and chromatographic conditions are listed below: (Table 2).

Samples were submitted to stability tests under a steady flow of O_2, N_2 and air, with and without a fluorescent light source (2150 lux).

Isotretinoin and its degradation compounds were analyzed by GC, following a series of preliminary chromatographic and thermal analysis assays, which allowed determination of GC parameters. Samples were evaluated after 1–15 days of exposure to the conditions listed above, and compared to USP reference standard for isotretinoin.

2.3. Thermal analysis

Thermal analysis of isotretinoin was performed at the Chemistry Institute—UNESP/Araraquara, in a SDT 2960-

Table 2
Chromatographic conditions for GC and HPLC system

GC system (1)	GC system (2)	HPLC system (1)	HPLC system (2)
007-65 HT column (15 m × 0.18 mm × 0.1 (m, Quadrex)	DB-WAX column (10 m × 0.1 mm × 0.2 (m, Sciences)	Lichrospher Si-60 column (100 mm × 4 mm)	Lichrospher Si-60 column (100 mm × 4 mm)
Inlet temperature: 185 °C;	Inlet temperature: 150 °C;	Mobile phase: hexane/ethyl acetate/dichloromethane (80:15:5) with 0.01% formic acid;	Mobile phase: hexane/ethyl acetate/dichloromethane (80:15:5) with 0.01% formic acid;
Detector temperature: 300 °C; Oven temperature: 90 °C (1 min) 10 °C/min 360 °C (7 min); Carrier gas: 0.4 mL/min H_2 flow Detection: FID	Detector temperature: 250 °C; Oven temperature: 90 °C (1 min) 10 °C/min 200 °C (8 min); Carrier gas: 0.4 mL/min H_2 flow; Detection: FID	Flow: 1 mL/min Detector: UV–vis (λ = 365 nm)	Flow: 1mL/min Detector: fluorescence (λ_{ex} = 350 nm; λ_{em} = 520 nm)

Table 3
Stablility tests under atmospheric and light stress conditions: stress conditions for samples

Sample	Atmospheric and Light stress conditions						Time
Raw material	Air		O_2		N_2		
Soft capsules (reference and generic brands)	Light	Dark	Light	Dark	Light	Dark	0, 1, 5, 10 and 15 days
Hard capsules	Air/light	Air/dark	O_2/light	O_2/dark	N_2/light	N_2/dark	

Simultaneous Analyzer, TA Instruments, at 100 mL min^{-1} of air, initial temperature of 30 °C, final temperature of 1000 °C with a heating rate of 10 °C min^{-1} and an isotherm at 1000 °C for 10 min.

2.4. Determination of GC parameters

Chromatographic parameters for the analysis of isotretinoin were established based on the study of the thermal behavior of the sample and the preliminary tests using several injector temperature to assess a possible degradative effect on the compound and the presence of residue on the injector.

Differential scanning calorimetry (DSC) and thermo gravimetric analysis (TG) were conducted to evaluate thermal behavior of isotretinoin raw material sample. Once the superior temperature limit was determined by thermal analysis, a series of chromatographic runs were performed to assess the existence of degradation products and the presence of residue in the injector during the analysis. For each test, the temperature of the injector was reduced in increments of 10 °C and subsequently in 5 °C.

The presence or absence of residue in the injector (memory effect) was confirmed by a subsequent run without introduction of sample in the system. Absence of residue was assumed when no peak was observed in the retention time expected for isotretinoin.

The tests to determine the injector temperature were performed with and without glass wool in the injector linner. These tests showed that glass wool had an adsorbent effect for isotretinoin. For this reason, further analyses were performed with injector temperature at 185 °C and without glass wool in the injector linner.

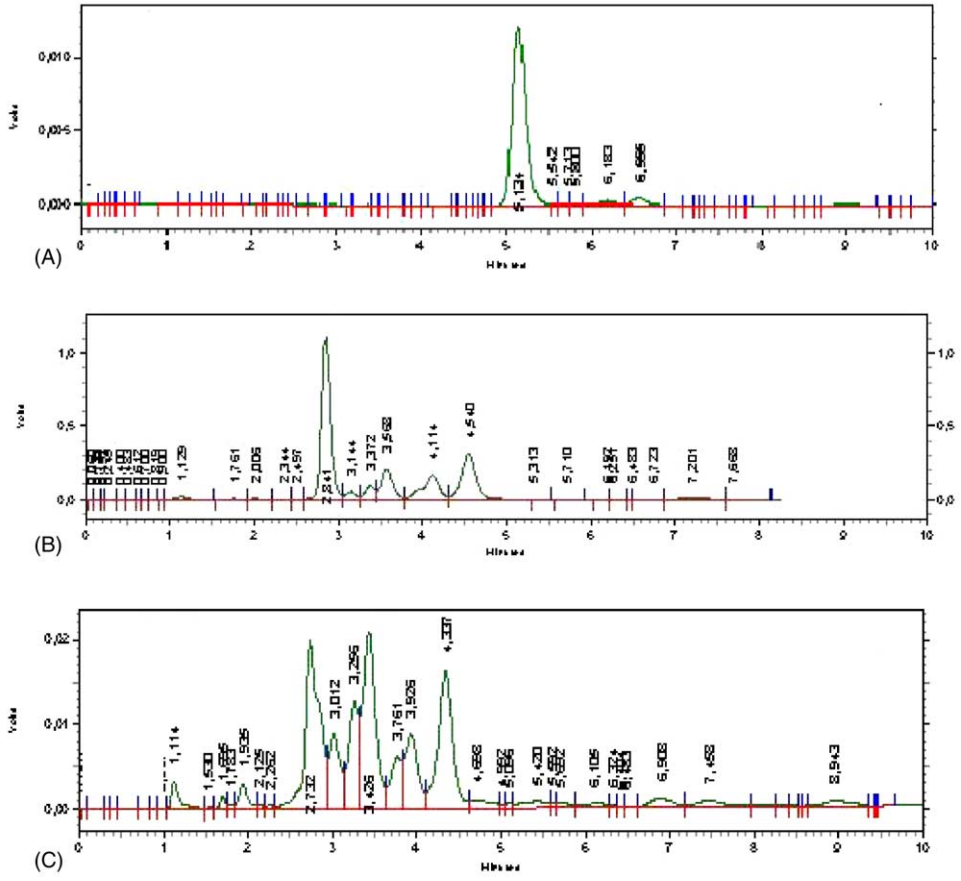

Fig. 1. (A) Chromatogram of isotretinoin RM, HPLC system (1); (B) chromatogram of soybean oil, HPLC system (1); and (C) chromatogram of isotretinoin RM, soybean oil added, HPLC system (1).

2.5. Preparation of samples

The whole procedure was performed taking into consideration that a high number of steps during the extraction process could increase degradation of isotretinoin. The contents of isotretinoin soft gelatin capsule were removed directly with the use of solvent and agitation. The capsules were opened with a sharp blade in a test tube containing dichloromethane in order to obtain a concentration of 2 mg mL^{-1} of isotretinoin in the solution.

2.6. Stability tests for isotretinoin

Stability tests were conducted under atmosphere and light stress conditions (Table 3). Isotretinoin was tested as samples of raw material, soft gelatin capsules, and hard gelatin capsules. Isotretinoin raw material was divided in 24 samples of 100 mg each and kept on PVC flasks. Twelve samples were submitted to the test in the dark and 12 were exposed to a 2150 lux fluorescent light, measured with a portable luximeter.

Each sample group was divided in three subgroups: one was connected to an oxygen flow line, the second was connected to a nitrogen flow line, and the third was exposed to the air for a period of 1–15 days.

3. Results and discussion

Several HPLC techniques described in the literature (Table 1), using different chromatographic systems (columns, flow rates, mobile phase combinations) were tested in this study, none of which was able to provide good separation and peak definition for all isotretinoin compounds. Unsatisfactory results using HPLC were observed in this work, particularly with pharmaceutical formulations of isotretinoin containing lipid excipients. In a 10-min chromatographic run, the isotretinoin peak was separated

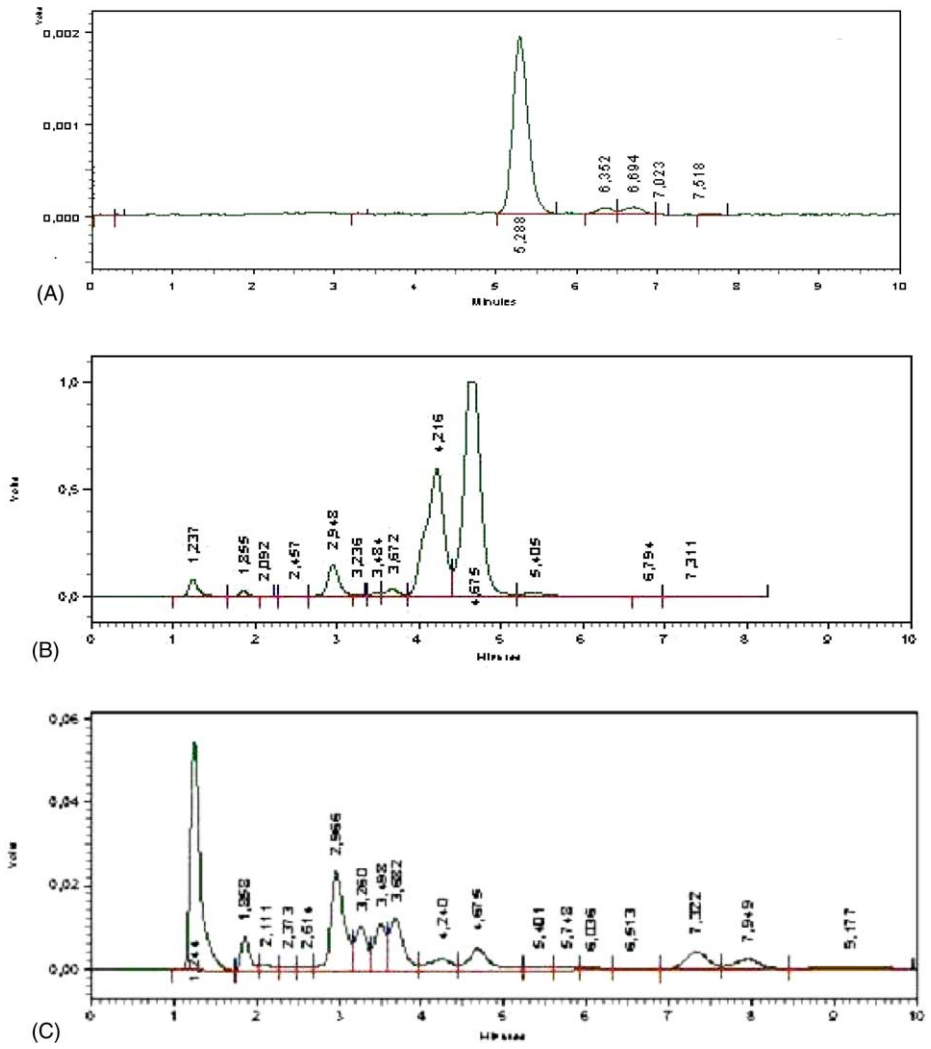

Fig. 2. (A) Chromatogram of isotretinoin RM, HPLC system (2); (B) chromatogram of soybean oil, HPLC system (2); and (C) chromatogram of isotretinoin RM, soybean oil added, HPLC system (2).

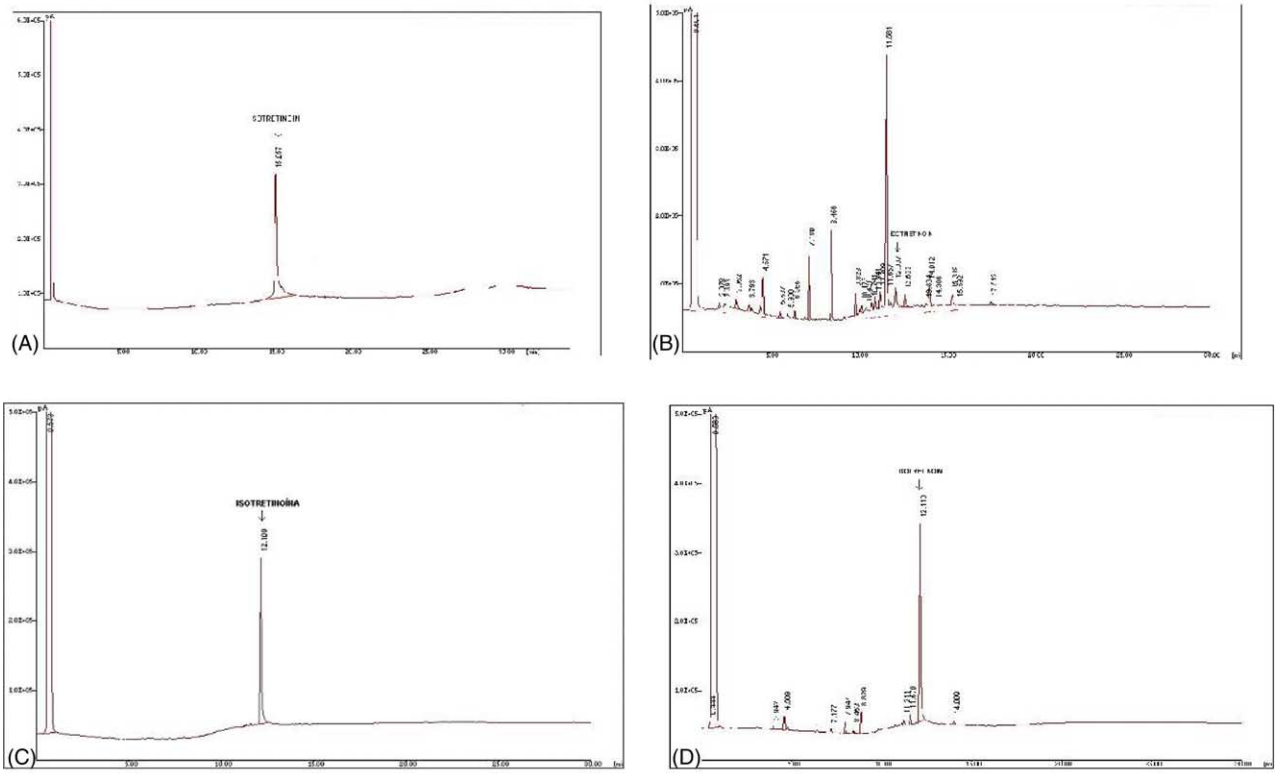

Fig. 3. Chromatograms of isotretinoin on GC system 2: (A) USP reference standard; (B) 15 days under O_2 flow and light; (C) 15 days under N_2 flow and dark; and (D) 15 days under O_2 flow and dark.

and identified at 5 min of elution time when isotretinoin raw material was tested. The same peak was not observed when soybean oil was added to the raw material (simulating commercial pharmaceutical formulations). The raw material with soybean oil exhibited others unidentified peaks in the separation area between the isotretinoin and soybean oil peaks (Figs. 1 and 2). This observation motivated the development of an analytical method for the analyses of isotretinoin by GC.

In this study, GC was used for the identification of isotretinoin in all samples tested in comparison with the standard reference sample (Fig. 3A). Preliminary thermal analysis test results showed that the sample undergoes mass loss

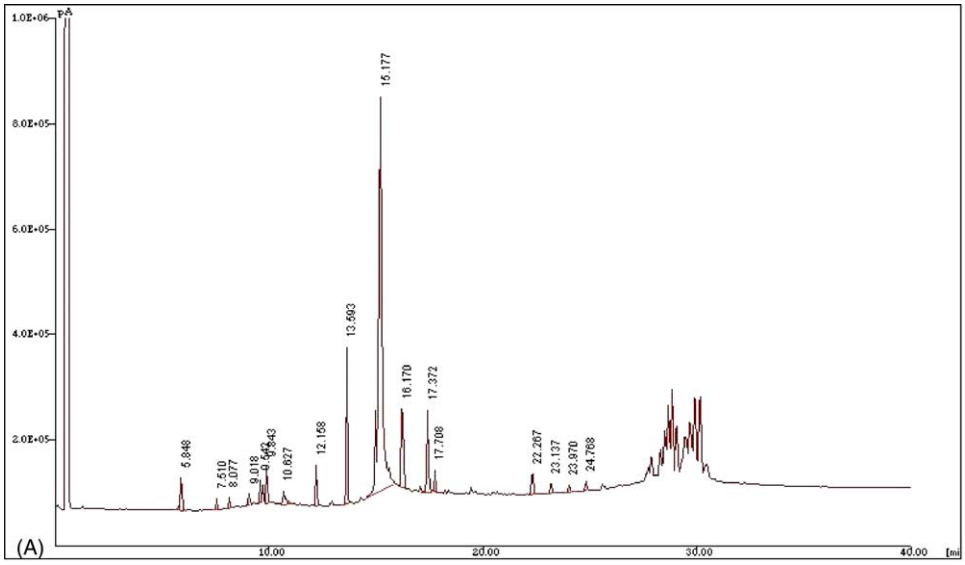

Fig. 4. Chromatogram of isotretinoin commercial pharmaceutical products, GC system (1): (A) generic brand; and (B) reference brand.

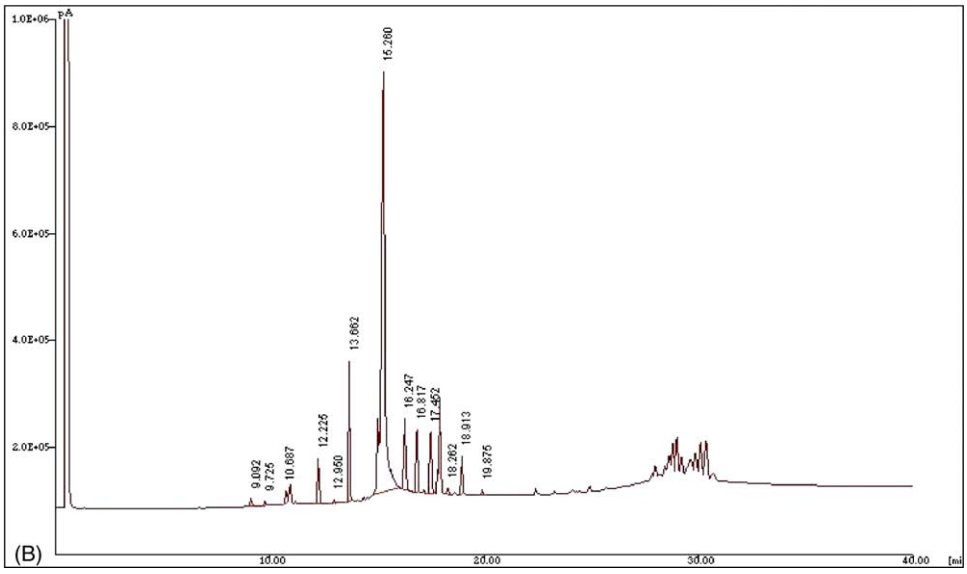

Fig. 4. (*Continued*).

starting at 200 °C, and the DSC curve shows a fusion peak at the same temperature. These results supported the establishment of the temperature parameters for further GC assays.

Isotretinoin and its degradation products could not be identified by HPLC, whereas GC showed excellent performance. Considering that the separation efficiency of GC was proven to be superior to that of HPLC, and that very little has been plubished on the use of GC to investigate isotretinoin in pharmaceuticals, the GC method was used for the analysis of isotretinoin samples submited to stability tests, using capillary columns of narrower diameter in comparison with traditional methods [29].

During stability studies, the samples exposed to O_2 or air under a fluorescent light, developed a high number of degradation products (Fig. 3B). In the same atmosphere conditions, but in the absence of light, the degradation profile was remarkably smaller (Fig. 3D). Samples under N_2 stream did not exhibit any degradation changes (Fig. 3C).

Qualitative changes could not be observed in the generic and reference brands of isotretinoin capsules. This was due to the presence of waxes, partially hydrogenated oils and other excipients, e.g. preservatives (Fig. 4). Further analyses using a mass detector for the identification of possible degradation products represents a good alternative considering that for both products severe physical–chemical changes were observed on the soft gelatin capsules after oxygen exposure.

4. Conclusions

Based on the results obtained from this work, it is possible to state that there is not one single method completely efficient for the analytical determination of all retinoids and their isomers. The use of capillary columns of narrow internal diameter and short length as well as an inert carrier gas instead of organic solvents, allowed better separation and definition of isotretinoin peaks and its degradation products.

As previously demonstrated by Tan et al. [15,16], and Botella-Muñoz et al. [30], the results obtained from stability and preliminary tests in this study, proved that fluorescent light enhances oxidative degradation reactions for this compound.

Both GC columns used (DB-WAX and 007-65HT) showed excellent separation efficiency. Even though DB-WAX column was able to separate isotretinoin a few minutes faster, 007-65HT column had the advantage of separating lipidic components present in the commercial samples.

This method for assaying isotretinoin is capable of separating and identifying this compound when in its isolated form and also when in a pharmaceutical commercial formulation. GC's separation efficiency superior to that of HPLC allows this method to be easily applicable to pharmaceutical samples, particularly for stability studies.

Acknowledgements

The authors would like to thank Prof. Massao from Ivo Giolito Laboratory and Prof. Dr. Maria Ines Leles for the help with the interpretation of thermal analysis results, TKS Farmaceutica, CAPES, CNPq and FINEP for financial support.

References

[1] M.B. Sporn, N.M. Dunlop, D.L. Newton, J.M. Smith, Proceedings of Federation, vol. 35, 1976, pp. 1332–1338.
[2] L.R. Harisiadis, R.C. Miller, E.J. Hall, C. Borek, Nature 274 (1978) 486–487.
[3] R. Lotan, Biochem. Biophys. Acta 605 (1980) 33–91.

[4] R. Lotan, D.E. Ong, F. Chytil, J. Natl. Cancer. Inst. 64 (1980) 1259–1262.
[5] T.R. Breitman, S.J. Collins, B.R. Keene, Blood 57 (1981) 1000–1004.
[6] M.B. Sporn, A.B. Roberts, Ciba Found. Symp. 113 (1985) 1–5.
[7] S. Nagpal, J. Athanikar, R.S.A. Chandraratna, J. Biol. Chem. 270 (1995) 923–927.
[8] R.A.S. Chandraratna, Br. J. Dermatol. 135 (1996) 18s–25s.
[9] M. Bakshi, S. Singh, J. Pharm. Biomed. 28 (2001) 1011–1040.
[10] TH.E. Gundersen, E. Lundanes, R. Blomhoff, J. Chromatogr. B 691 (1997) 43–58.
[11] TH.E. Gundersen, R. Blomhoff, J. Chromatogr. A 935 (2001) 13–43.
[12] R. Gatti, M.G. Gioia, V. Cavrini, J. Pharm. Biomed. Anal. 23 (2000) 147–159.
[13] P.A. Lehman, A.M. Malany, J. Invest. Dermatol. 93 (1989) 595–599.
[14] C. Echoff, H. Nau, J. Lipid. Res. 31 (1990) 1445–1454.
[15] X. Tan, N. Meltzer, S. Lindenbaum, Pharm. Res. 9 (1992) 1203–1208.
[16] X. Tan, N. Meltzer, S. Lindenbaum, J. Pharm. Biomed. Anal. 11 (1993) 817–822.
[17] V. Berbenni, A. Marini, A. Cardini, Int. J. Pharm. 221 (2001) 123–141.
[18] R. Gatti, M.G. Gioia, A.M. Di Pietra, M. Cini, J. Chromatogr. A 905 (2001) 345–350.
[19] P.A. Lehman, T.J. Franz, J. Pharm. Sci. 85 (1996) 287–290.
[20] P. Lefebvre, A. Agadir, M. Cornic, B. Gourmel, B. Hue, C. Dreux, L. Degos, C. Chomienne, J. Chromatogr. B 666 (1995) 55–61.
[21] G. Caviglioli, B. Parodi, S. Caffagi, G. Bignardi, G. Romussi, Drug Dev. Ind. Pharm. 20 (1994) 1295–1408.
[22] R. Wyss, F. Bucheli, J. Chromatogr. B 700 (1997) 31–47.
[23] B. Disdier, H. Bun, J. Catalin, A. Durand, J. Chromatogr. B 683 (1996) 143–154.
[24] D.K. Bempong, I.L. Honigberg, N.M. Meltzer, J. Pharm. Biomed. Anal. 13 (1995) 285–291.
[25] M. Miyagi, H. Yokoyama, H. Shiraishi, M. Matsumoto, H. Ishii, J. Chromatogr. B Biomed. Sci. Appl. 757 (2001) 365–368.
[26] E.S.M. Po, J.W. Ho, B.Y. Gong, J. Biochem. Biophys. Meth. 34 (1997) 99–106.
[27] M. Brisaert, J. Plaizier-vercammen, Int. J. Pharm. 199 (2000) 49–57.
[28] B.R. Simmons, O. Chukwumerije, J.T. Stewart, J. Pharm. Biomed. Anal. 16 (1997) 395–403.
[29] A.R. Oyler, M.G. Motto, R.E. Naldi, K.L. Facchine, P.F. Hamburg, D.J. Burinsky, R. Dunphy, M.L. Cotter, Tetrahedron 45 (1989) 7679–7694.
[30] S. Bottela-Muñoz, M.A. Martín, B. Castillo, D.A. Lerner, J.C. Menéndez, Anal. Chim. Acta 468 (2002) 161–170.