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Aerosol based detectors for the investigation of phospholipid hydrolysis in a pharmaceutical suspension formulation

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ABSTRACT

A high performance liquid chromatography (HPLC) method was developed to quantify free fatty acids (FFA) in a pharmaceutical suspension formulated with phospholipids as stabilizing agents. Specifically, a suspension of crystalline itraconazole microparticles stabilized with Lipoid E80 was used as a model system to study the physicochemical stability of an aqueous, phospholipid-based suspension for injection. The hydrolysis of the phospholipids during storage at elevated temperatures (40 °C) necessitated the development of a suitable HPLC method for the determination of free fatty acid content in the suspension samples.

HPLC methods using two types of aerosol detectors were investigated for the above purpose. Reversed-phase separation coupled with either an evaporative light scattering detector (ELSD) or a Corona^{Plus} charged aerosol detector (CAD) was used. A comparison of the methods indicated that the CAD method provided better sensitivity, precision, recovery, and linearity for the parameters evaluated. As a result, this method was chosen for the stability study of itraconazole suspension and has been incorporated in subsequent formulation studies.

1. Introduction

Phospholipids are well-known as stabilizing agents for various types of dispersed-phase pharmaceutical formulations including emulsions, liposomes, solid lipid nanoparticles, and suspensions [1–4]. Their history of demonstrated biocompatibility and safety, and thus their acceptance by regulatory agencies, has led to their widespread use. Phospholipids are known to undergo both oxidation and hydrolysis, and therefore, the impact of these degradation processes on the physicochemical stability of the pharmaceutical formulation must be assessed. Although oxidation can be mitigated through the addition of antioxidants or by atmospheric control, hydrolysis can be prevented only by removal of water from the system.

A suspension of crystalline drug microparticles stabilized with phospholipids was studied in our laboratory to determine the effect of phospholipid hydrolysis on the physical and chemical stability of the formulation. The antifungal agent itraconazole was used as the model drug, and a commercially available egg lecithin (Lipoid E80) was used as the stabilizing agent. The primary components of Lipoid E80 are phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which hydrolyze to form Lyso-PC and Lyso-PE, respectively, and free fatty acids. Therefore, as a part of this investigation, a suitable analytical technique was required to characterize five long-chain fatty acids that are the major products of Lipoid E80 hydrolysis. They are three unsaturated (linolenic, linoleic, oleic) and two saturated (palmitic, stearic) fatty acids.

Fatty acids are traditionally analyzed by gas chromatographic (GC) techniques and in most cases pre-column sample derivatizations are required to improve volatility and detectability of the analytes [5,6]. Methods using HPLC with ultraviolet (UV) detection have also been reported. However, these methods also require pre-column derivatization to improve sensitivity since these compounds lack adequate UV absorbance [7–9]. Capillary electrophoresis (CE) with indirect photometric detection is another technique reported previously [10]. For the determination of free fatty acids in food and blood, an enzymatic assay has also been reported [11]. Although the GC and HPLC methods are widely used routinely, these methods are not applicable for itraconazole suspension matrix due to the negligible aqueous solubility of the drug.

HPLC coupled with aerosol detectors are typically used for the detection of lipids lacking an adequate UV chromaphore; therefore, such techniques are especially suited to free fatty acids analyses. An aerosol detector that has gained great popularity since the 1980s is the evaporative light scattering detector (ELSD).

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This detector is popular for detecting non-UV absorbing analytes and is recommended highly as an alternative to refractive index (RI) detection, since RI can only work under isocratic mobile phase conditions. The theory and operation of ELSD has been described previously [12–14]. It is widely used for specific applications such as in lipids, carbohydrates, surfactants, polymers, amino acids and pharmaceuticals. However, ELSD has certain limitations; for example, in pharmaceutical analysis it often produces different responses for compounds of the same molecular weight. As a result, ELSD can be unsuitable for impurity determination in drug material. Lack of sensitivity for certain compounds and non-linearity for wide ranges are other problems associated with ELSD.

Recently, the Corona charged aerosol detector (CAD) was introduced as an answer to the quest for better sensitivity that was lacking with ELSD [15]. The principle of CAD is based on charging of aerosol particles by corona discharge and subsequent measurement of the charged particles using an electrometer, similar to an atmospheric chemical ionization mass spectrometry (APCI/MS). The principle and operation of CAD has been reported previously [15,16].

Both CAD and ELSD can operate under similar mobile phase conditions; hence transferring methods from ELSD to CAD is effortless. Volatile mobile phases and non-volatile or semi-volatile analytes are required for both detection methods. For ELSD, aerosol particles are detected by light scattering, which depends on the size of the particle. This can affect the sensitivity and precision of the method, since not all the particles are produced uniformly. For CAD, the response is based on electrical aerosol detection technology, which has been demonstrated to be sensitive even for very small particles (<100 nm) [15,17]. Higher sensitivity for various ranges of particle sizes also provides better reproducibility and linearity over wide ranges. CAD also has the advantage of lower operational cost, as it does not require optical components for detection as in the case of ELSD.

HPLC methods for FFA analysis using ELSD and CAD were developed in our laboratory and the results of specificity, sensitivity, precision, recovery, and linearity were compared. Typical ICH validation parameters for impurity determination in drug products were used as guidance for the method qualification [18]. Comparison of ELSD vs. CAD for FFA determination and the application of the latter for investigation of FFA formation in itraconazole samples kept at elevated temperature are described in this paper.

2. Materials and methods

2.1. Reagents

Itraconazole suspension was prepared using itraconazole purchased from DSM Pharma (Parsippany, NJ, USA) and Lipoid E80 from Lipoid GmbH (Ludwigshafen, Germany). Lipoid E80 contains 80.0–85.0% phosphatidylcholine (PC) + Lyso-phosphatidylcholine (LPC), 7.0–9.5% phosphatidylethanolamine (PE), and 2.0–3.0% sphingomyelin. The amount of LPC is not more than 3.0% and the amount of free fatty acids is not more than 0.05% (information provided by manufacturer). 1N sodium hydroxide was purchased from Ricca (Arlington, TX, USA) and was used for pH adjustment. Free fatty acids (Linolenic, linoleic, palmitic, oleic and stearic) for standard and spiked sample preparation were purchased from Sigma–Aldrich (St. Louis, MO, USA).

HPLC grade acetonitrile, chloroform and methanol were purchased from Burdick & Jackson (Muskegon, MI, USA) for the mobile phase and sample diluent preparation. Acetic acid (glacial, 99% purity) was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA).

2.2. Itraconazole suspension preparation

Itraconazole suspension was prepared at a nominal concentration of 1 wt% by dispersing itraconazole powder in an aqueous phase consisting of 2.4% Lipoid E80 using an IKA Ultra-Turrax T25 mixer (Staufen, Germany). The suspension was then homogenized using an Avestin EmulsiFlex-C55 piston-gap homogenizer (Ottawa, Canada) to achieve micron-sized particles. The pH of the suspension was adjusted to 9.40.

2.3. Standards and sample preparation

For the initial stages of method development using the ELSD system, calibration standards of FFA (linolenic, linoleic, palmitic, oleic, stearic) were prepared at 10, 25, 50, 75, 100, 120 and 150 μg/ml in a sample diluent (50:50 mixture of 70/30 methanol/chloroform and 0.1% acetic acid in acetonitrile). These standards were used for generating quadratic plots. Later this method was improved by using a logarithmic plot, in which case only standards at 10, 100 and 150 μg/ml were prepared. These standards were used for both ELSD and CAD methods.

Itraconazole suspension samples were spiked with various levels of FFA and further diluted with sample diluent to prepare for HPLC analysis. The concentrations of FFA in the spiked samples were 10, 50, 100, 120 and 150 μg/ml. These solutions were used for precision, recovery and sample linearity determination.

2.4. Apparatus

The HPLC/ELSD system consisted of an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled with an Alltech ELSD 2000ES detector (Grace Davison, Deerfield, IL, USA). The nitrogen gas (ultra-pure >99%) used to operate ELSD and CAD systems was produced using nitrogen generators manufactured by Grace Davison (Deerfield, IL, USA). Separation was performed on a 150 mm × 3.0 mm Zorbax SB-C18, 3.5 μm column (Agilent Technologies, Palo Alto, CA, USA).

The HPLC/CAD system consisted of a Waters 2695 HPLC system (Milfred, MA, USA) and a Corona^{Plus} CAD^{TM} (ESA Biosciences, Inc., Chelmsford, MA, USA). Waters Empower^{TM} software (Milford, MA, USA) was used to collect all the data.

2.5. Stationary phase and mobile phase conditions

Separation conditions for both ELSD and CAD systems were the same. Free fatty acids were separated on a Zorbax SB C18 column (150 mm × 4.6 mm) containing 3.5 μm particles. The mobile phase contained 20% water, 80% acetonitrile and 0.1% acetic acid. The flowrate was maintained at 1.0 ml/min under isocratic conditions. The column and sample temperatures were kept at 30 °C and 5 °C, respectively. The injection volume was 15 μl.

2.6. CAD and ELSD parameters

The gas pressure for CAD was kept at 35 psi and the detector temperature was maintained at 40 °C. The current range of the CAD was 100 pA. The ELSD was operated in impactor 'ON' mode with a gas flowrate of 1.2 ml/min. The ELSD drift tube temperature was kept at 30 °C.

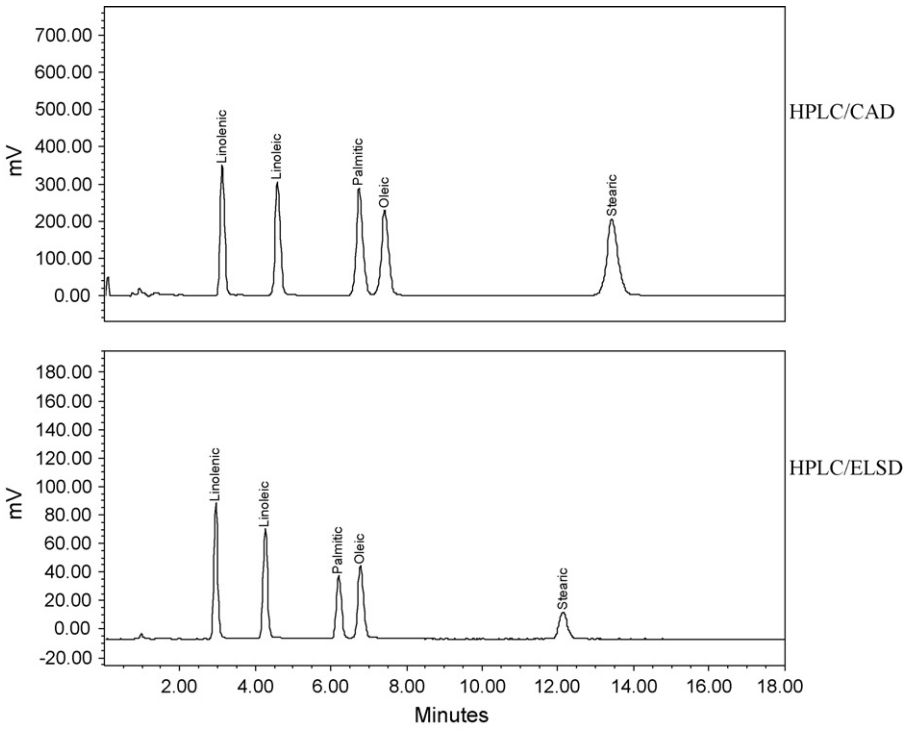

Fig. 1. Chromatograms of FFA by HPLC/CAD and HPLC/ELSD.

3. Results and discussion

3.1. Method development

Separation of FFA required a stationary phase that could withstand low pH mobile phase conditions and the Zorbax stable bond (SB) C18 column was chosen to meet this requirement. The packing material is not endcapped, and as a result it provided superior column stability and reproducibility for all the analytes. The mobile phase used for the separation contained 0.1% acetic acid in 20/80 water/acetonitrile. Acetic acid is commonly used as an organic buffer in the mobile phase when aerosol detectors are used, where mobile phase volatility is a fundamental requirement. The calculated pH of 0.1% acetic acid is 3.27, well below the pK_a of all the acids. At this pH, acidic analytes behave as neutral species, increasing the hydrophobicity of the compounds and thus their retention time. Use of high percent (80%) organic phase improved the solubility of FFA under low pH conditions. The flow rate was kept at 1.0 ml/min regardless of the small particle size and the internal diameter, allowing overall analysis time to be less than 15 min.

Standards of 100 μg/ml FFA were made and injected on both systems to evaluate the chromatographic profile. Fig. 1 shows typical chromatograms of five different FFA standards with CAD and ELSD both. All FFA were eluted within 15 min under both conditions. Peak heights of FFA are at least 4 times higher with the CAD method. It is worth noting that the retention times of all five fatty acids are quite different even though the carbon chain length for all but palmitic acid are the same. This concurs with the several previous reports that separation of FFA is not only affected by carbon chain length, but also depends on the degree of unsaturation [19,20].

3.2. Qualification of the method

3.2.1. Specificity

A matrix solution containing only itraconazole was prepared in the sample diluent (50/50 mixture of 70/30 methanol/chloroform and 0.1% acetic acid in acetonitrile) and injections of this matrix solution, sample diluent, mobile phase, and FFA standards were performed to assess specificity. Upon evaluation of the chromatograms, specificity was confirmed for all but linolenic acid (chromatograms not shown). Due to the high signal of itraconazole in the matrix solution chromatogram, a slight rise in the baseline was observed at the retention time of the linolenic acid peak on both systems. As a result, linolenic acid was excluded from the stability evaluation of itraconazole suspension.

3.2.2. Precision and accuracy

Itraconazole suspension samples spiked at various concentrations of FFA were analyzed to determine method accuracy and precision. Concentrations of FFA in the spiked samples ranged from

Table 1
Accuracy and precision—HPLC/CAD.

Sample	% Recovery linolenic	% RSD linolenic	% Recovery linoleic	% RSD linoleic	% Recovery palmitic	% RSD palmitic	% Recovery oleic	% RSD oleic	% Recovery stearic	% RSD stearic
Spike-10 $N=3$	106.1	0.6	108.5	1.1	99.5	1.2	108.0	1.0	98.8	1.7
Spike-50 $N=3$	105.3	1.4	105.6	0.5	100.8	1.1	106.7	1.0	104.7	2.0
Spike-100 $N=6$	97.1	2.2	100.0	1.8	102.6	0.9	102.7	1.3	103.4	0.4
Spike-120 $N=3$	97.1	2.2	97.3	2.2	100.3	1.5	98.3	3.0	99.6	0.8
Spike-150 $N=3$	90.8	2.4	92.6	2.1	97.2	2.1	94.0	1.9	96.8	1.4

Table 2
Accuracy and precision—HPLC/ELSD.

Sample	% Recovery linolenic	% RSD linolenic	% Recovery linoleic	% RSD linoleic	% Recovery palmitic	% RSD palmitic	% Recovery oleic	% RSD oleic	% Recovery stearic	% RSD stearic
Spike-10 $N=3$	98.5	4.4	94.1	3.9	102.0	9.0	93.2	1.1	104.2	11.2
Spike-50 $N=3$	101.9	1.2	97.6	0.7	86.6	0.6	97.7	0.2	80.4	2.9
Spike-100 $N=6$	108.4	4.7	102.7	0.7	102.0	2.0	99.7	0.7	95.8	2.1
Spike-120 $N=3$	104.3	2.0	100.4	1.3	105.1	1.4	99.6	1.0	97.9	4.9
Spike-150 $N=3$	106.3	3.4	103.7	1.3	109.3	0.6	103.3	0.2	101.0	0.8

10 to 150 μg/ml. Triplicate injections of each preparation were made except for the 100 μg/ml spiked sample, which was injected six times.

The results from the precision and accuracy study are shown in Tables 1 and 2. The percent RSD ranged from 0.4 to 3.0% for the CAD method, and from 0.2 to 11.2% for the ELSD method. Though the aerosol formation mechanism is the same for both detectors, ELSD results were less precise, especially at lower concentrations. The differences in precision between the detectors arise from the fundamental differences in their detection methods. ELS detection is susceptible to poor precision at low concentrations, due to Mie scattering caused by small analyte particles [13,21]. Since CAD is based on electrical charging of the particles, followed by electrometer detection, it is well-known for higher sensitivity of low concentration analytes and more uniform behavior for a wider range of samples [16,17]. This results in better reproducibility in most cases.

Accuracies of the methods were determined by calculating the percent recoveries of the analytes in the spiked samples, based on their theoretical concentrations. Expected recoveries were to be within the range of 90–110%, typical for impurity determination in drug products. For the CAD, this expectation was met since the recoveries ranged from 90.8 to 108.5% while the ELSD results were less accurate as the recoveries ranged from 80.4 to 109.3%.

3.2.3. Linearity

To evaluate linearity of the ELSD system, seven calibration standards ranging 10 to 150 μg/ml were initially analyzed. The concentrations were plotted against their corresponding peak areas. The performance of ELSD is known to be non-linear [13,22–24]. Hence, a quadratic relationship was fitted with R^2 values ranging from 0.98 to 0.99 (data not shown). An alternate approach to solve the problems of laborious standards preparation and data processing that are typically associated with the quadratic calibration plots is the use of log–log plots [24,25]. Calibration curves were constructed by plotting logarithm of analyte concentrations against the logarithm of peak areas. Using this approach, only a three-point calibration curve was needed throughout the analyses instead of the seven-point curve used in the initial stages of method development.

The CAD response was also non-linear; therefore, log–log plots were also used to create calibration curves for the optimized methods for this system. The resulting calibration data for both systems are shown in Table 3. In all the cases, CAD results were superior to that of ELSD. To demonstrate the sample linearity of spiked sam-

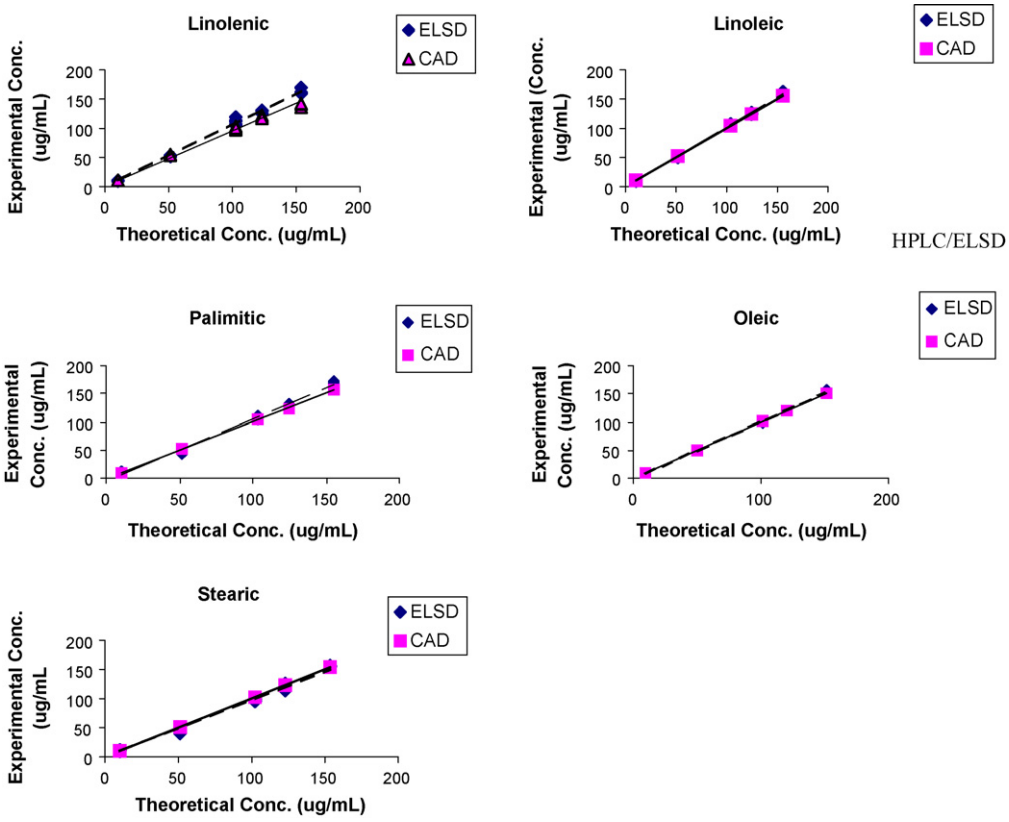

Fig. 2. Calibration plots in the range of 10, 50, 100, 120 and 150 μg/ml of spiked samples—CAD vs. ELSD.

Table 3
Calibration and LOD data—CAD vs. ELSD.

FFA	CAD			ELSD		
	Equation	R^2	LOD (μg/ml)	Equation	R^2	LOD (μg/ml)
Linolenic	Y = 8.38e−001X + 4.73e+000	0.999	0.26	Y = 1.39e+000X + 3.02e+000	0.988	0.96
Linoleic	Y = 8.56e−001X + 4.72e+000	0.999	0.30	Y = 1.36e+000X + 3.04e+000	0.981	1.1
Palmitic	Y = 8.79e−001X + 4.76e+000	1.000	0.38	Y = 1.35e+000X + 2.91e+000	0.994	1.8
Oleic	Y = 8.92e−001X + 4.68e+000	0.999	0.41	Y = 1.32e+000X + 3.06e+000	0.981	1.7
Stearic	Y = 9.79e−001X + 4.64e+000	1.000	0.75	Y = 1.31e+000X + 2.91e+000	0.989	3.4

Table 4
Free fatty acids amount in itraconazole formulation stored at 40 °C.

Fatty acid (μg/g)	Stability time points		
	Initial	28 Days	63 Days
Linoleic	ND	297.0	662.8
Palmitic	ND	957.0	2102.4
Oleic	ND	704.0	1770.6
Stearic	ND	439.4	1009.3

ND = not detected.

ples, theoretical concentrations of FFA in spiked samples (10, 50, 100, 120 and 150 μg/ml) were plotted against their corresponding experimental concentrations. As illustrated in Fig. 2, comparable linearity was achieved for both ELSD and CAD, demonstrating linearity throughout the range even in the presence of the itraconazole matrix.

3.2.4. Limit of quantification (LOQ) and limit of detection (LOD)

For CAD, the LOQ for all FFA was determined to be 10 μg/ml, the lowest concentration FFA in the sample linearity curve that gave acceptable precision and accuracy results. However, with ELSD, the precision for stearic acid recoveries was higher (11.2%). This finding is consistent with previous reports, in which CAD was proven to provide better S/N ratio values for various lipids at low concentrations, whereas ELSD responses are better at high concentrations [21]. Additionally, at least in two instances (palmitic and stearic), the recoveries were below 90% for the ELSD method. As a result, the LOQ determination for these two FFA using ELSD was inconclusive.

The limits of detection (LOD) values were estimated using serially diluted standards and were determined as the lowest amount injected that provided peaks with signal-to-noise ratios of 3 or greater. The results from this study are listed in Table 3. In all cases, the limits of detection for CAD were approximately four times lower than that for ELSD.

Upon completion of the evaluation studies of both methods, we chose the HPLC/CAD method for the screening of itraconazole suspension stability. Although both methods could be used to quantify the five FFA of interest in this study, the CAD method was more sensitive, more accurate and more precise compared to the ELSD method. The responses of both methods are non-linear; however, by plotting the calibration functions on a logarithmic scale, linearity was achieved.

3.3. Investigation of phospholipid hydrolysis in itraconazole formulation

Investigation of phospholipid hydrolysis in the itraconazole formulation was performed using the HPLC/CAD method. Itraconazole formulation samples stored at 40 °C for 63 days were tested at three different time points. Linolenic acid was not included in this study because of the interference from the high concentration of itraconazole in the matrix. Linoleic, palmitic, oleic and stearic acids were characterized and the results are shown in Table 4. Initially (T = 0), the free fatty acids content was below the detection limit. However, the levels of FFA increased significantly over the 63 days storage period. The results indicate that degradation of PE and PC occurred, resulting in the formation of FFA. This was substantiated by a significant decrease in pH, as well as by a reduction in the concentrations of PE and PC and an increase in Lyso-PC over time. Therefore, the suitability of HPLC/CAD method for the quantification of FFA due to phospholipid hydrolysis in itraconazole formulation was demonstrated.

4. Conclusion

Two types of aerosol based detectors were used in conjunction with HPLC for the determination of free fatty acids in an itraconazole suspension formulation. Results from the comparison studies were evaluated in terms of specificity, precision, accuracy, linearity, recovery and sensitivity. In all cases, the results obtained by the HPLC/CAD were superior to the HPLC/ELSD results. As a result, the HPLC with CAD was chosen for the investigation of phospholipid hydrolysis in the itraconazole formulation. This method is a valuable tool for the screening of phospholipid-based formulations and has been incorporated in various formulation development studies.

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