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DEVELOPMENT OF A SIMPLE HPLC METHOD USING CORE-SHELL PARTICLES COLUMN FOR QUANTIFICATION OF IR3535® IN INSECT REPELLENT LIQUID FORMULATIONS

Sinh Thai Nguyen[1], Jean-Paul Salvi[1], Elodie Gilbert[2], Celine Serre[2], Fabrice Pirot[2], Roselyne Boulieu[1*]

[1]Clinical Pharmacy, Pharmacokinetics, and Drug Evaluation Laboratory, [2]Industrial Pharmaceutics Laboratory, EA4169 "Normal and pathological functions of the cutaneous barrier", Faculty of Pharmacy, Claude Bernard Lyon 1 University, University of Lyon, 8 avenue Rockefeller, 69373 Lyon Cedex 08, France

***Corresponding author e-mail:** roselyne.boulieu@univ-lyon1.fr

ABSTRACT

A simple, fast, precise and sensitive method for the quantification of IR3535®, an insect repellent used in topical formulations, was developed by reversed-phase high performance liquid chromatography. The separation was carried out by using a core-shell silica particles column (Kinetex® C18, 5μm, 4.6 mm x 150 mm) with a mobile phase consisting of 0.1% formic acid and methanol (40 : 60 v/v) in an isocratic mode at 0.5 ml/min and UV detection at 220 nm. The calibration curve was linear from 10 to 400 μM, with regression coefficient r^2 = 0.9999. Limit of detection (LOD) and Limit of quantification (LOQ) were found to be 1 and 10 μM, respectively. Precision and accuracy fulfilled the acceptance criteria. This method was validated and may be routinely used for quantitative analysis of IR3535® from various topical insect repellent liquid formulations.

Keywords: Insect repellent · IR3535 · HPLC · Validation

INTRODUCTION

Mosquitoes and ticks are common vectors of numerous infection diseases in human. Their saliva injected during blood feeding can cause swelling and irritation, as well as transport of viruses, bacteria and vector-borne pathogens[1,2]. Thus currently, USA and other countries in Europe have approved the use of repellents as an important tool to protect people from these infections[3]. Repellents are chemical substances that alter insect flight behavior and inhibit these pathogen vectors from landing on the skin and clothes[4,5]. Ideally, repellents should protect against biting insects with a long-lasting effectiveness and cause no adverse reactions[6]. Published data indicate that repellent efficacy and protection duration vary considerably among products and among mosquito or tick species[7-9]. It is also significantly affected by other factors such as ambient temperature, level of human activity, amount of perspiration, or exposure to water[10,4]. Several insect repellents were evaluated and registered by the U.S. Environmental Protection Agency for skin application, such as DEET (N,N-diethyl-meta-toluamide), Picaridin, IR3535® or Oil of Lemon Eucalyptus (OLE)[11].The most widely marketed chemical-based insect repellent is DEET, which is effective against many species of mosquitoes, biting flies and ticks. Although known as the "standard" repellent, DEET has problems with safety due to its high skin permeability, unpleasant odors and several side effects[4,12,13]. Moreover, recent studies have revealed that some individual insects are becoming increasingly resistant to DEET[12]. Besides, IR3535® is a good DEET alternative with the same advantages and fewer disadvantages. Developed in the early 1970s, IR3535®, chemically ethyl butylacetylaminopropionate or 3-(N-acetyl-N-butyl) aminopropionic acid ethyl ester (Figure-1), is derived

from a natural amino acid, β-alanine[14,3]. Despite its marketed history of 25 years in european countries, this chemical has just been introduced as a product in USA from 1999[3]. As well, the active ingredient is not under patent.

In comparison with other common insect repellents, IR3535® has very low toxicity at equal efficacy[15]. This chemical has been tested for a number of toxic risks and showed accepted side effects. Even though eye irritant and sometimes skin irritant may occur, it still exhibits an excellent skin tolerance and general safety[3,16]. In 2001, the World Health Organization declared this repellent safe and effective for human use[17].

The repellent effects of IR3535® and DEET against mosquitoes (*Culex, Anopheles*species) have shown comparable protection efficacy[18]. Depending on mosquito species and testing method, IR3535® has demonstrated widely variable effectiveness with complete protection times ranging from 23 minutes to over 10 hours[1,3,7,19,20]. It also provides up to 12 hours of protection against blacklegged ticks[7].

Due to its interesting cosmetic properties (non-sticky, odorless), IR3535® can be used in various cosmetic formulations, such as aqueous alcoholic lotions, oil-in-water or water-in-oil emulsions, pump sprays, aerosols, roll-ons, gels, and creams[21,15].

Repellent products containing alcohol are thought to be able to permeate deeper into the skin, which results in a faster loss of effectiveness[4] and the rapid absorption of dermally applied IR3535® may lead to toxicity[14]. Therefore, our research group has investigated to develop an alcohol-free formulation of IR3535® that allows better efficacy and safety.

Despite currently used in over 150 consumer products worldwide, there are few documents related to the validation of a IR3535® quantitative method reported in the literature[16]. Thus, the aim of the present study was to develop and validate a simple and reliable HPLC method for quantification of IR3535®assay from a topical repellent formulation.

MATERIALS AND METHODS

Apparatus: The liquid chromatographic system included a Waters Alliance 2795 pump, an auto injector and a Waters 2996 UV DAD detector (Waters Corporation, USA). Integration of the detector output was performed using the Waters Empower®2 software (Waters Corporation, USA).

Chemicals and reagents: IR3535® was received from the Merck company. The formulations containing 8% or 10% (w/w) IR3535® were supplied by pharmaceutics laboratory (ISPB, Claude Bernard Lyon 1 University, Lyon, France) and were stored at room temperature. Methanol and formic acid of HPLC-quality grade were purchased from Merck (Darmstadt, Germany). Water purified through a Purelab® Option purifying system (ElgaLabWater) was used in all procedures.

HPLC conditions: Analysis of IR3535® was performed under isocratic elution, using a Kinetex® column, C18, 5μm, 4.6mm × 150mm (Phenomenex, USA). The mobile phase consisted of 0.1% formic acid (pH = 2.89) and methanol in a ratio of 40:60 v/v. The run time was set up at 10.0 min. Analysis was executed at room temperature (25±1^{o}C). Prior to injection, the column was equilibrated for 30 min with the mobile phase. Detection was set at 220 nm with a flow rate of 0.5 mL.min^{-1}and the volume of injection was 15 µL.

Stock and working solutions: The stock solution was obtained by dissolving 22 μL of IR3535® with purified water in a 250 mL flask to obtain a final concentration of 400 μM. From the stock solution, working standard solutions of IR3535® were prepared in the range of concentration from 10 to 400 μM to establish the calibration curve as well as to evaluate the precision and accuracy of the method. Dilutions were performed with water.

Preparation of alcohol-free formulation of IR3535®: The formulation was designed as alcohol-free lipidic nanoparticle liquid suspension. IR3535®was dispersed into oily phase consisted of different waxes, a solubilizing agent and surfactants. This oily phase is dispersed into an aqueous phase consisted of only water or water associated with a thickening agent.

Preparation of sample solution: To quantify IR3535® in a topical formulation, samples required dilution up to 1000-fold or 2000-fold (for formulation containing 8% or 10% IR3535®, respectively) with regard to the linear range of the calibration curve. First, acetonitrile was added into a 5 mL flask contained 0.5 mL of the liquid formulation. Sample was then vortexed 5 min and centrifuged 10 min at 3000 rpm, 20^{o}C. Supernatant was filtered through a 0.20 μm PTFE membrane (Phenomenex, USA). Subsequently, 1 mL of this solution was diluted 10 fold with mobile phase and vortexed 2 min. Finally, the above solution was diluted 10-fold or 20-fold with mobile phase for 8% and 10% IR3535®

formulations respectively and vortexed 2 min. The last solution was then injected into the column.

METHOD VALIDATION

The validation of the chromatographic method was carried out according to the procedures described in the International Conference on Harmonization (ICH) guidelines[22].

(a). Linearity: Linearity of proposed analytical method describes the relationship between analyte concentration in the sample and the peak area. In our study, the linearity was evaluated by analyzing IR3535® solutions at six concentration levels (10, 25, 50, 100, 200, 400 µmol/L) and in quadruplicate (n=4). Calibration curve was obtained from the least-squares regression model. For acceptance, a correlation coefficient (r^2) of 0.997 or better was required.

(b). Precision: Repeatability (intra-day) was obtained by measuring five replicates of quality control samples at 300, 150, 25 µM and the limit of quantification (LOQ) on the same day. Intermediate precision (inter-day) was verified by repeating the above procedure on five different days. Intra-day and inter-day precision were presented as relative standard deviation (RSD). The precision around the mean value should be equal or inferior to 15% for normal concentrations and to 20% for LOQ.

(c). Accuracy: Accuracy was expressed as a percentage error (PE) from the theoretical concentration. PE should not exceed 15% for the quality control samples, except for the LOQ which should not exceed 20%[23,24].

(d). Limit of detection (LOD): To determine the LOD, the graphic approach was used. A signal-to-noise ratio of 3:1 is considered acceptable for estimating the detection limit[22].

(e). Limit of quantification (LOQ): LOQ was determined during precision and accuracy evaluation. The acceptance criteria of these two parameters at LOQ are 20% for both RSD and PE. The LOQ was used as the smallest concentration on the calibration curve[23].

RESULTS AND DISCUSSION

Method development: The aim of this work was to develop a simple and reproducible analytical method to determine the amount of IR3535® in topical formulations. Currently, only two publications related to the analysis of IR3535® were reported. One was about the determination of IR3535® in biological samples from human subjects after dermal application[14]. A ReproSil Pur ODS3 column (150 x 2 mm, 5µm) was used. Mobile phases consisted of 0.1% formic acid : methanol 40:60 (v/v) in a gradient elution mode. Flow rate is only 200µL/min but retention time of the compound of interest is more than 14 minutes. A triple stage quadrupole mass spectrometer was needed for quantification of IR3535® in biological materials.

The other method proposed consists in the determination of IR3535® in an insect repellent gel[16]. A Hypersil ODS-C18 column, (250 x 4.6mm, 5µm) was used. The mobile phase was water: acetonitrile 40:60 (v/v), using gradient elution mode and UV detection. Retention time of IR3535® was 8 minutes with a flow rate of 1.0mL/min.

In our study, a Kinetex® C18 column (150 x 4.6mm, 5µm) was chosen. This stationary phase exhibited less band broadening compared to fully porous particles and thus results in better resolution, higher sensitivity and low back pressure [25]. The optimal mobile phase composition was confirmed to be 0.1 % formic acid and methanol (40:60, v/v) as reported in the first paper[14]. However, even using a simple isocratic elution mode, IR3535® was eluted in a shorter retention time (6.2 minutes). The choice of methanol versus acetonitrile contributes to reduce the cost of the analysis.

As shown in Figure-2 corresponding to the typical chromatogram of the repellent formulation containing 8% of IR3535®, no interference of any excipients which are included in the formulation was observed. Compared to the only HPLC method reported in the literature for analysis of IR3535® in a gel formulation[16], the method described is simpler and cheaper due to short time analysis and the use of methanol instead of acetonitrile.

Method parameters: A typical calibration curve within a range from 10 to 400 µM (n = 6) was obtained by the following linear regression line: $y = 3803.3x - 11218$ with a high correlation coefficient (r^2 = 0.9999) (Figure-3). Intra-day and inter-day precision and accuracy are shown in Table-1 and Table-2. All values fulfilled acceptance criteria proposed by the ICH guidelines. As shown in table 1 and table 2, RSD and PE were 6.60% and 6.68% for intra-day and 7.47% and 11.39% for inter-day measurements, respectively for a concentration of 10 µM. The LOQ was defined as 10 µM. LOD was found at 1 µM.

CONCLUSION

The described HPLC method using core-shell silica particles as stationary phase provided satisfactory outcomes with symmetric peak, high resolution from excipients in the formulation and low back pressure. We have succeeded in finding out a suitable, simple and rapid HPLC method using an isocratic mode. With results of linearity, precision, and accuracy, this method may be adequately used for quantitative routine analysis of IR3535® in topical insect repellent liquid formulations.

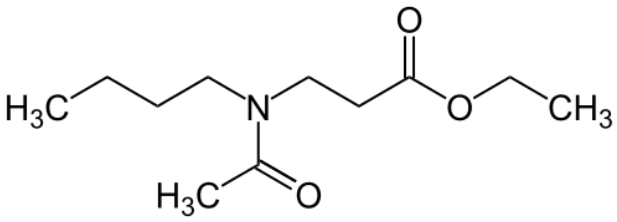

Fig-1: Structure of IR3535®

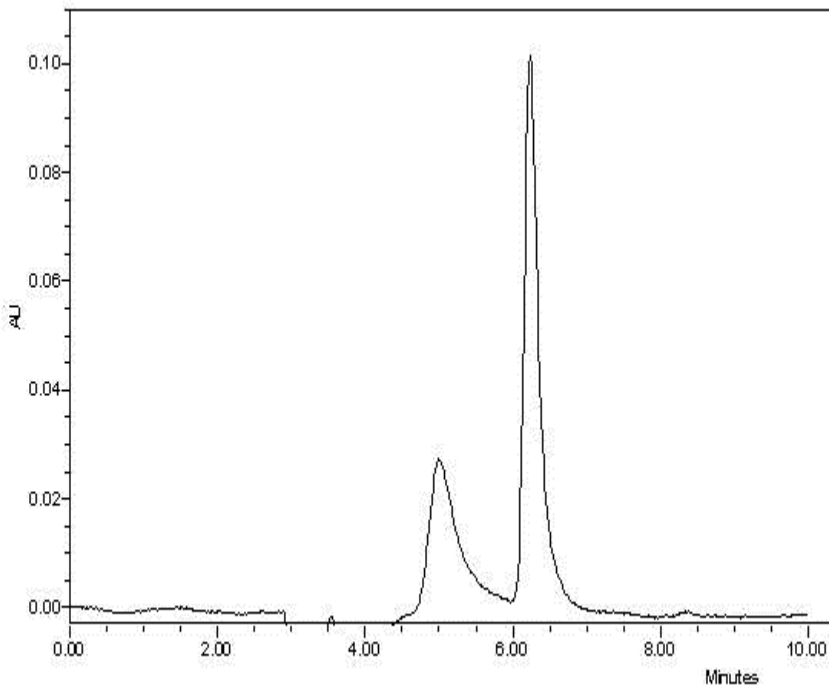

Fig-2: Chromatogram of IR3535® (t_R=6.2mn) in a topical 8% formulation.

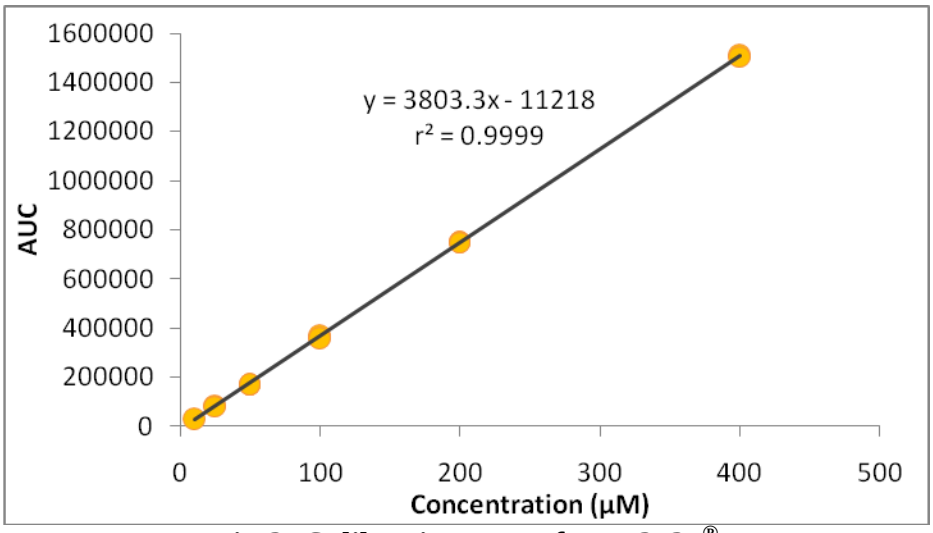

Fig-3: Calibration curve for IR3535®

Table-1: Intra-day precision and accuracy (n=5)

Theoretical conc. (µM)	Mean ± SD (µM)	RSD (%)	PE (%)
10	10.67 ± 0.70	6.60	6.68
25	25.30 ± 0.49	1.93	1.19
150	146.31 ± 1.71	1.17	2.46
300	296.31 ± 3.19	1.08	1.23

Table-2: Inter-day precision and accuracy (n=5)

Theoretical conc. (µM)	Mean ± SD (µM)	RSD (%)	PE (%)
10	11.14 ± 0.83	7.47	11.39
25	25.50 ± 0.83	3.27	2.01
150	143.67 ± 4.20	2.92	4.22
300	296.11 ± 3.11	1.05	1.30

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