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Short communication

Development and validation of a reversed-phase high-performance liquid chromatographic method for the determination of ethyl-3-(*N*-*n*-butyl-*N*-acetyl)aminopropionate in an insect repellent semi-solid formulation

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Abstract

A reversed-phase high-performance liquid chromatographic method with detection at 220 nm was developed and validated for the determination of ethyl-3-(*N*-*n*-butyl-*N*-acetyl)aminopropionate, IR 3535, in an insect repellent semi-solid product. A Hypersil ODS RP-C_{18} column (250×4.6 mm), 5 μm particle size, was equilibrated with a mobile phase consisted of water–acetonitrile (60:40, v/v). Its flow-rate was 1.0 ml/min. Excipients did not interfere with the determination of IR 3535 ($R_s = 8.663$). Intra- and inter-day relative standard deviations for samples were not higher than 0.61 and 1.2%, respectively. Mean recovery was found not lower than 98.5% and not higher than 100.3%. The method of external standard was adopted. Calibration curves were linear in the concentration range between 1.0×10^{-6} and 5.0×10^{-4} *M*. Limits of detection and quantitation were 65 and 196 ng/ml, respectively.

Keywords: Validation; Ethyl-3-(*N*-*n*-butyl-*N*-acetyl)aminopropionate; IR 3535; Insect repellents

1. Introduction

Insect repellents [1–5] are substances which cause the alteration of insect flight path. This is the basic difference between repellents and insecticides. The former substances prevent insects from landing on their potential target [6,7] while the latter kill them.

The most important requirements of an insect repellent are effective protection of the skin from insects, long-lasting repellent action, maximum skin tolerance without toxic or allergic properties, no skin penetration, high chemical stability under use conditions, good aesthetic reactions and acceptable cost per use of the final product. A new insect repellent produced by RONA/Merck (Darmstadt, Germany), IR 3535, with the chemical name ethyl-3-(*N*-*n*-butyl-*N*-acetyl)aminopropionate (Fig. 1), fulfills all the above requirements. In comparison with other common insect repellents on the market [8–13], IR 3535 shows the advantage of very low toxicity at equal efficacy (Merck's Technical Report). Moreover, there is no analytical method reported in the literature for its determination in any kind of sample.

The purpose of the present work was to develop a simple, fast, sensitive and reproducible, reversed-phase HPLC method for the determination of IR

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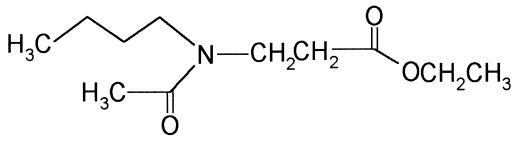

IR 3535

Fig. 1. Structure of ethyl-3-(*N*-*n*-butyl-*N*-acetyl)aminopropionate, IR 3535.

3535 in an insect repellent semi-solid formulation for topical application. In this paper, development, optimization and validation of such a method is presented.

2. Experimental

2.1. Instrumentation

The chromatographic system used, consisted of a Waters 600E multisolvent delivery system (a 600 controller, a pump and a U6K injector) and a Waters 486 tuneable absorbance detector (Waters, Milford, MA, USA). The above system was controlled by the software package Millennium 2010. The pH of the mobile phase was measured with a pH Meter 3310 Jenway (Gransmore Green, UK).

2.2. Chemicals and reagents

All chemicals were of analytical purity grade. Acetonitrile (ACN) of HPLC grade was purchased from E. Merck (Darmstadt, Germany). Insect repellent IR 3535 of technical purity grade, samples of insect repellent gels and their excipients of analytical purity grade were kindly donated by the pharmaceutical company Lavipharm (Peania, Attica, Greece). Water purified with a Milli-Q RG water purification system (Millipore, Bedford, MA, USA) was used in all procedures.

2.3. Chromatographic conditions

A reversed-phase Hypersil ODS-C_{18} column (250×4.6 mm, 5 μm particle size) was used. The mobile phase consisted of water–acetonitrile (40:60, v/v). The mobile phase was degassed for 10 min with helium gas at a degassing rate of 20 ml/min. The flow-rate of the mobile phase was 1.0 ml/min. Injection volume was 20 μl. Experiments were performed at ambient temperature. Absorption was measured at 220 nm. The elution time of IR 3535 was less than 8.5 min.

2.4. Solution preparation

2.4.1. Stock solutions

First, 21.6 mg of IR 3535 and 134.6 mg of insect repellent gel were dissolved in 100.0 ml of mobile phase and 50.0 ml of purified water, respectively. Concentration of the resulting stock solutions was about 1.00×10^{-3} *M*. The nominal concentration of IR 3535 in the samples under study was 8% (w/w). Stock solutions were stored at −20 °C and were stable for at least a week.

2.4.2. Test solutions

Working standard solutions of IR 3535 were prepared in the concentration range from 1.0×10^{-6} to 5.0×10^{-4} *M* and used for the establishment of the linearity range, the construction of calibration curves and evaluation of the precision of the proposed method. Dilutions to volume were performed with mobile phase.

2.4.3. Sample preparation

Determination of IR 3535 in insect repellent samples was performed by calibration curves. An amount of 1 ml of stock solutions of samples was diluted to 10 ml with mobile phase so that the final expected concentration of IR 3535 in the injected solutions was approximately 1×10^{-4} *M*. Recovery studies of the developed method were performed in one sample using the method of standard additions. A series of four solutions was prepared. The first solution, contained only insect repellent sample (expected concentration 8.0×10^{-5} *M*), was prepared as described above. The other three contained, in addition to insect repellent sample, increasing amounts of standard solution of IR 3535, 5.0×10^{-5}, 1.00×10^{-4} and 2.00×10^{-4} *M*, respectively. The prepared solutions were then injected to the HPLC system.

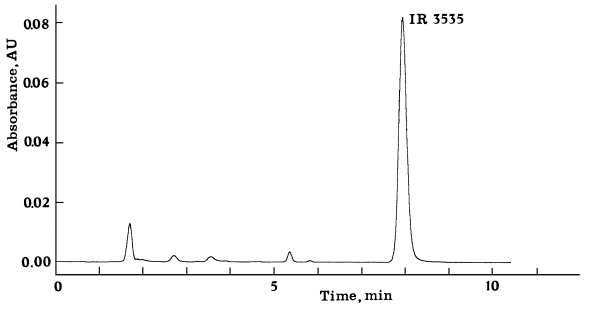

Fig. 2. Chromatogram of an insect repellent gel in which the nominal concentration of IR 3535 was 3.7×10^{-4} *M*. The chromatographic conditions used were: ODS-C_{18} column, mobile phase water–acetonitrile (60:40, v/v), flow-rate 1.0 ml/min, detection wavelength 220 nm and room temperature.

2.5. Data analysis

Calibration curves of IR 3535 were constructed for its determination in insect repellent semi-solid products. Regression equations were obtained through unweighed least squares linear regression analysis, applied to IR 3535 peak areas as a function of its concentration.

3. Results and discussion

3.1. Mobile phase

Several eluent mixtures were tried and it was noticed that the more polar the mobile phase, the longer the retention time of IR 3535 was. A mobile phase consisting of water–acetonitrile (60:40, v/v) was preferred over another one containing water–acetonitrile (50:50, v/v) because the former resulted in a more symmetric IR 3535 peak as indicated by the calculated values of the asymmetry factor: $A_{s,f}$ (60:40) = 1.06 and $A_{s,f}$ (50:50) = 1.29. Resolution was good in both cases: R_s (60:40) = 8.663±0.028 and R_s (50:50) = 4.773±0.027.

3.2. Selectivity

A typical HPLC chromatogram of the insect repellent gel is shown in Fig. 2. This sample, apart from its active ingredient, IR 3535, included several excipients. These substances were provided by Lavipharm for a study of the selectivity of the developed method. Each substance was injected in the chromatographic system, after being dissolved and diluted with mobile phase. It was proved that none of the excipients interfered with the determination of IR 3535 under the experimental conditions used. Good resolution for IR 3535 and the nearest excipient peak was assured by the value of R_s which was 8.663±0.028.

3.3. Calibration curve of IR 3535

Under the experimental conditions described in Sections 2.3 and 2.4.2, linear calibration curves were obtained throughout the examined concentration range of IR 3535. Regression analysis of peak areas of IR 3535 (*y*) versus its concentration (*x*) was carried out. The results are tabulated in Table 1.

Table 1
Analytical parameters of the calibration curve and the method of standard additions of IR 3535

Method	Concentration range ($\times10^4$ *M*)	Regression equation[a]		
		Intercept (a±SD)[b]	Slope (b±SD[b])$\times10^{-7}$	r (n)[c]
Calibration curve	0.1–5.0	−1156±1624	268.9±1.0	0.99998 (7)
Standard addition[d]	–	229 700±3213	266.8±2.8	0.9999 (4)

The chromatographic conditions used were: ODS-C_{18} column, mobile phase water–acetonitrile (60:40 v/v), flow-rate 1.0 ml/min, detection wavelength 220 nm and room temperature.

[a] Linear unweighed regression analysis, with a regression equation $y = a + bx$, where x is concentration in M.

[b] SD is the standard deviation of intercept and slope.

[c] r is the correlation coefficient and n is the number of points in each calibration curve; each point is the mean of four experimental measurements.

[d] The estimated concentration of IR 3535 in the sample was approximately 8×10^{-5} *M*, while standard additions of IR 3535 were 5.0×10^{-5}, 1.00×10^{-4} and 2.00×10^{-4} M.

Preparing and measuring standards of the same concentration of IR 3535 four times each, relative standard deviation (RSD) was calculated and found less than 0.46% in the whole concentration range.

3.4. Precision and accuracy

To verify the precision of the proposed HPLC method, intra- and inter-day precision of standards and samples was obtained. The relative standard deviation of IR 3535 standards and samples was not higher than 0.46 and 0.61% for within-day and 1.0 and 1.2% for between-day measurements, respectively.

The accuracy of the developed method was examined by recovery studies conducted as described in Section 2.4.3. The mean recovery of IR 3535 was calculated and found not lower than 98.5% and not higher than 100.3%. The results of the recovery studies of IR 3535 are summarized in Table 2.

3.5. Limits of detection (LOD) and quantitation (LOQ)

The LOD was defined as the analyte concentration that gives a signal equal to $y_b + 3.3s_b$, where y_b is the signal of the blank and s_b is its standard deviation. Similarly, the LOQ was defined as $y_b + 10s_b$. In the unweighed least-squares method is quite suitable in practice to use $s_{y/x}$ [14] instead of s_b and the value of the calculated intercept a instead of y_b. Thus:

$$\text{LOD} = \frac{3.3s_{y/x}}{b} \quad \text{and} \quad \text{LOQ} = \frac{10s_{y/x}}{b}$$

where b is the slope of the regression line. Based on the above equations, the calculated LOD value for IR 3535 was 65 ng/ml, while the LOQ was 196 ng/ml.

3.6. Determination of IR 3535 in insect repellent gel

There was no official or other method found in the literature for the determination of IR 3535 in any kind of sample. An internal standard was sought in the beginning. However, the method of external standard was finally adopted because the latter proved to be simple, precise and accurate without complicated sample preparation that would have necessitated use of an internal standard. In contrary, addition of an internal standard would have definitely increased the elution time of chromatograms because of the existence of several excipients in the samples under study. Instead, the method of standard additions was applied to check the accuracy of the obtained results in one sample. Unweighed least-squares linear regression analysis was applied and the results of the standard addition method were included in Table 1, along with those taken by the calibration curve for comparison reasons. It was observed that the slopes of both curves were statistically the same. This meant that there was no interference of the matrix in the determination of IR 3535 in the sample [15] and the developed method was accurate.

Six samples of a gel with different lot numbers, were dissolved in purified water and diluted with mobile phase as described in the sample preparation section. The nominal mass ratio of IR 3535 in this product was 8% (w/w). Results of this analysis were reported in Table 3.

Table 2
Recovery studies for the determination of IR 3535

Added concentration of IR 3535 ($\times 10^4$ M)[a]	Mean recovery±SD (%)[b]
0.5	99.6±0.7
1.0	100.3±0.1
2.0	98.5±0.4

The chromatographic conditions used were: ODS-C_{18} column, mobile phase water–acetonitrile (60:40, v/v), flow-rate 1.0 ml/min, detection wavelength 220 nm and room temperature.

[a] The nominal concentration of IR 3535 in the control sample was 8.0×10^{-5} M.

[b] SD is the standard deviation of the mean recovery.

Table 3
Determination of IR 3535 in insect repellent gel

Sample	Nominal concentration of IR 3535 (%, w/w)	Concentration found±SD[a] (%, w/w)	
		Intra-day	Inter-day
1		8.13±0.02	8.14±0.08
2		7.94±0.02	7.96±0.07
3	8.0	8.23±0.04	8.21±0.08
4		8.23±0.05	8.23±0.09
5		8.33±0.01	8.34±0.10
6		8.10±0.01	8.09±0.09

The chromatographic conditions used were: ODS-C_{18} column, mobile phase water–acetonitrile (60:40, v/v), flow-rate 1.0 ml/min, detection wavelength 220 nm and room temperature.

[a] SD is the standard deviation of the mean % (w/w) concentration found.

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