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Development of a HPLC method for the determination of antichagasic phenylethenylbenzofuroxans and its major synthetic secondary products in the chemical production processes

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Abstract

A simple isocratic reverse-phase HPLC method for the determination of six antichagasic phenylethenylbenzofuroxans and its major synthetic secondary products, the corresponding geometric isomers and the benzofurazans, was developed and validated for use in the analysis of pre-clinical studies. Separation was achieved on a reverse-phase Supelco LC-18 column using either methanol–acetonitrile–water or acetonitrile–water, in different proportions, as mobile phase. The compounds were eluted isocratically at a flow rate of either 0.8 or 1.0 mL min^{-1}. The compounds were analyzed with UV detection at 210 and 300 nm. The validation characteristics included linearity, accuracy, precision, specificity, limit of detection and quantification and robustness. Validation acceptance criteria were met in all cases. This method was used successfully for the quality assessment of the drugs production in the scale-up procedures.

Keywords: Phenylethenylbenzofuroxans; HPLC; Secondary product; Geometric isomers; Benzofurazans

1. Introduction

Chagas' disease or American trypanosomiasis is an important health problem that affects around twenty million people in Central and South America. Around 2–3 million individuals develop the typical symptoms of this disease that results in 50 000 yearly deaths [1,2]. The causative agent of this disease is the haemoflagellate protozoan *Trypanosoma cruzi* (*T. cruzi*). As part of our research program involving new agents against *T. cruzi* phenylethenylbenzofuroxan derivatives have been identified as excellent lead compounds [3–9]. They have also been shown to be non-toxic against mammal cells [10]. The urgent necessity of new antichagasic drugs conducts us to introduce these compounds in a pre-clinical study program ongoing a "Drugs for Neglected Diseases *initiative*" project [11]. For this purpose we have selected the most *in vitro* selective compounds, derivatives **1**–**6** (Fig. 1), in order to study its *in vivo* activities, mutagenic profiles and mammal metabolic behaviors. These derivatives were synthesized in a kilogram-process involving an olefination Wittig-Boden's methodology (Fig. 1). In these reaction conditions were generated both stereoisomeric form of the desired compounds, namely *E*- and *Z*-geometric isomers. Eventhough Boden's mild conditions were used, the corresponding deoxygenated products, benzofurazans **7**–**12** (Fig. 1), were identified as reaction by-products. When the batches of reactions were HPLC-monitored, the chromatographic profile, as it is exemplified in Fig. 2a, showed the presence of at least three different compounds. However, batch reaction's ^{1}H NMR spectra showed the presence of four different entities, the expected two benzofuroxans' geometric isomers and the corresponding by-products benzofurazans in both geometric isomeric forms.

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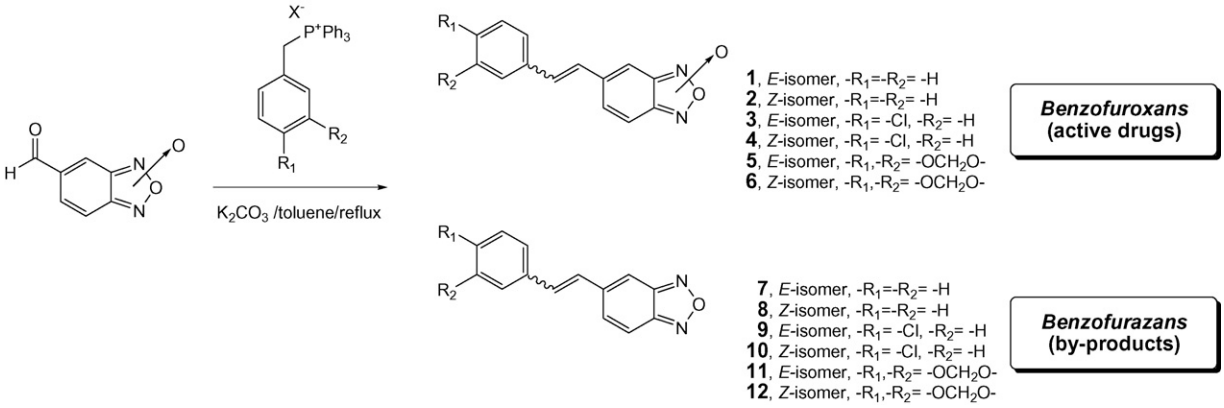


Fig. 1. Kilogram-procedure employed in the production of phenylethenylbenzofuroxans with anti-*T. cruzi* activity.

In the scale-up production procedure each desired products, **1**–**6**, was isolated using column chromatography and purified by successive crystallizations [12] (Scheme 1). After chromatographic separation each desired products was contaminated with the corresponding deoxygenated analogue. For example, derivative **4** was contaminated, according to HPLC analysis and confirmed by ^{1}H NMR experiments, with 2% of benzofurazan **10** (Fig. 6b). The benzofurazan analogues, **7**–**12** (Fig. 1), resulted *in vitro* inactive against the different assayed strains of *T. cruzi* [8,10] focusing great attention on the need to monitor the presence of them in the scale-up final product. Regarding phenylethenylbenzofuroxans and its synthetic by-products there are not analytical methods described in the literature for its quantitative determination. To the best of our knowledge there also are not analytical methods for benzofuroxans. Hence, an attempt has been made to develop a simple, efficient and selective method for the determination of phenylethenylbenzofuroxans and its major synthetic by-products in the selected kilograms-synthetic procedures. HPLC instrumentation with UV detection was used. The total analysis run time is less than 20 min. The method was used successfully to evaluate the progress of the synthetic procedures as well as the concentration and quality of the final products.

2. Experimental

2.1. Samples and chemicals

All the studied compounds, **1**–**12**, and its intermediates produced in multi-step synthetic reaction were obtained in our laboratory according to previously published methods [8,10]. The final products, **1**–**6**, were isolated first by chromatographic column and then purified by successive crystallizations in the adequate solvent (Table 1) until acceptable microanalyses (C,H,N). The samples homogeneity was achieved by NMR spectroscopy and microanalysis. As internal standard was used 4-nitroanisole purchased from Merck Co. Nylon syringe filters were purchased from Sartorius AG, Göttingen, Germany. HPLC grade methanol and acetonitrile were purchased from J.T. Baker and Fisher Scientific, respectively. HPLC deionized water (18 MΩ) was obtained from a Milli-Q purification system, Millipore S.A.

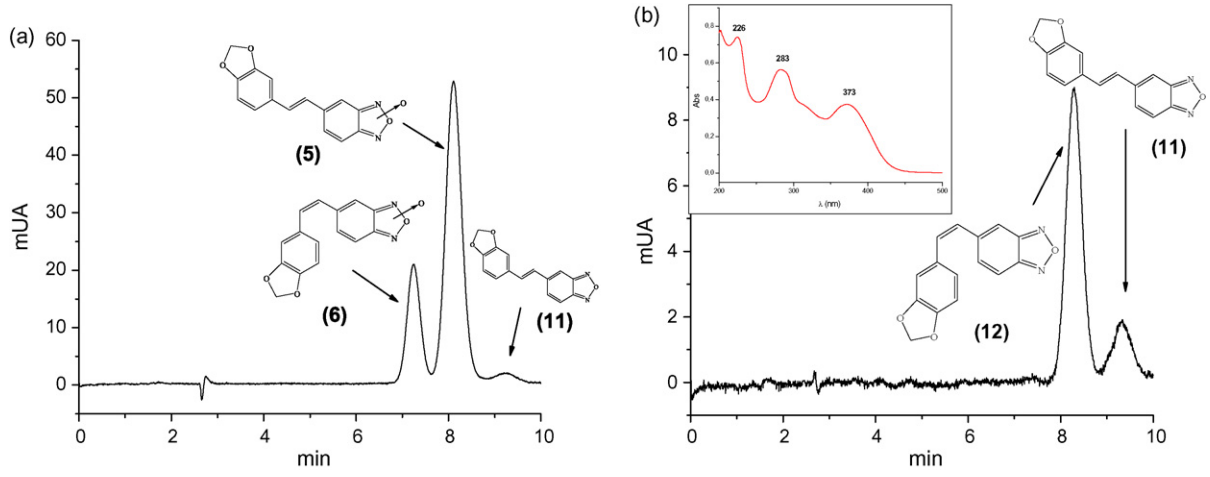
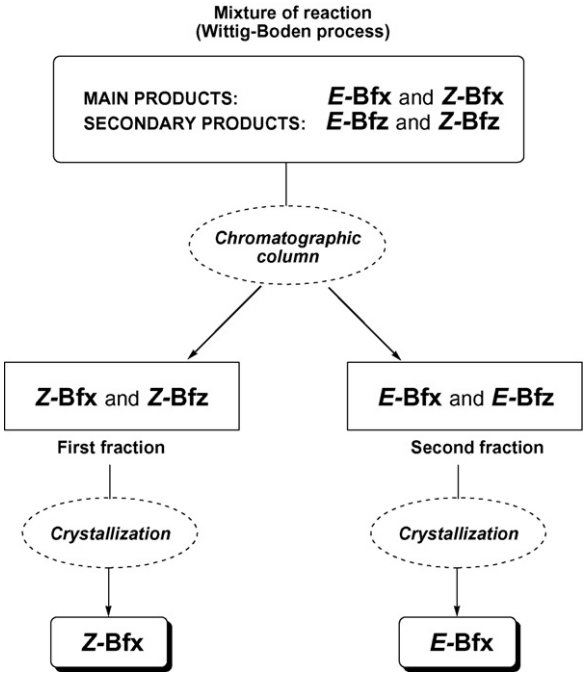


Fig. 2. (a) HPLC profile of the synthetic batch for derivatives **5** and **6** production. (b) HPLC profile of derivatives **5** and **6** synthetic by-products, deoxygenated analogues **11** and **12** respectively. *Inset*: sub-product **12** UV spectrum (in CH_3CN). Run conditions: reverse-phase Supelco LC-18 column, isocratic elution with mobile phase of acetonitrile:water, 40:60 (v/v), column temperature 27 °C, injection volume 10 μL, flow rate 1.0 mL min^{-1}, UV detection at 300 nm.



Scheme 1. Schematic procedure developed to isolate and purify the desired benzofuroxans geometric isomers. Bfx: benzofuroxan, Bfz: benzofurazan.

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of a PerkinElmer LC-135C/LC-235C, Series 410 LC BIO PUMP equipped with a quaternary pump, online degasser, column heater, autosampler and diode array-detector. Data collection and analysis were performed using Turbochrom Navigator Workstation software (Version 6.1.2; PerkinElmer Instruments, LCC). Separation was achieved on a Supelco LC-18 column, 250 mm × 4.6 mm I.D., particle size 5 μM, (Supelco Inc., Bellefonte, PA, USA). The elutions were isocratics with mobile phase and flow rates reported in Table 1. The column temperature was maintained at 27 °C. The injection volume was 50 μL with UV detection at 210 and 300 nm. Drug concentration was assessed by measuring chromatographic peak areas.

2.3. Preparation of standard solutions

The stock solutions of the analytes were prepared in the mixture of solvent indicated in Table 1. All the individual standards solutions were found to be stable for at least 1 month when stored at ≤4 °C. Linearity-studies standard solutions at six levels, precision- and accuracy-studies standard solutions at three levels were prepared by serially diluting the stock solutions with the HPLC mobile phase to concentrations shown in Tables 4 and 5.

2.4. Method validation

The method was validated according to the United States Pharmacopeia (USP) requirements [13,14]. The following validation characteristics were addressed: linearity, accuracy, precision, specificity, limit of detection and quantification and robustness. These studies were performed using as detection λ = 300 nm. The UV profiles of the analytes **1**–**6** were analyzed (Fig. 3). Some response differences, between the geometric isomers, were observed when λ = 210 and 300 nm were used in the HPLC detection, see example in Fig. 4, however for all the studied compounds λ = 300 nm was the adequate wavelength for the simultaneous determination of each desired product, geometric isomers, and its secondary products.

2.4.1. System suitability standard

System suitability standard solutions containing the mixture of stereoisomers (10 μM of each isomer), namely **1** and **2**, **3** and **4**, and **5** and **6**, were prepared by diluting and mixing the stock solutions with mobile phase. These solutions were found to be stable for at least 1 month when stored at ≤4 °C. System suitability was determined from six replicate injections of the system suitability standard before sample analysis. The acceptance criterion for each stereoisomer and for the mixture is gathered in Table 2. Resolution was calculated using the following equation: $Rs = 1.18\ [(t_2 - t_1)/(W_2 + W_1)]$ where t_2 and t_1 are the retention times (RT) of each stereoisomers and W_2 and W_1 are the peak widths at half height [15]. The results were used to monitor critical operational parameters of the chromatographic system to confirm that the resolution and precision were adequate immediately prior to analysis.

2.4.2. Linearity

Standard calibration curves were prepared with six calibrators (three times each) over different concentration ranges. The data peak areas versus drug concentrations

Table 1
Characteristics of the samples and selected chromatographic conditions

Compound	Crystallization solvent (mp, °C)	HPLC parameters		
		Mobile phase (v/v)	Flow rate (mL min^{-1})	Stock solution solvent (v/v)
1	Petroleum ether:EtOAc, 85:15 (144.0–145.0)	MeOH:CH_3CN:H_2O, 45:5:50	0.8	MeOH:H_2O, 50:50
2	Petroleum ether (62.0–63.0)	MeOH:CH_3CN:H_2O, 45:5:50	0.8	MeOH:H_2O, 50:50
3	EtOH (173.0–174.0)	CH_3CN:H_2O, 40:60	1.0	CH_3CN:H_2O, 40:60
4	Petroleum ether:EtOAc, 90:10 (116.0–117.0)	CH_3CN:H_2O, 40:60	1.0	CH_3CN:H_2O, 40:60
5	EtOH (183.7–184.5)	MeOH:CH_3CN:H_2O, 5:35:60	1.0	CH_3CN:H_2O, 40:60
6	Petroleum ether:EtOAc, 90:10 (98.5–100.3)	MeOH:CH_3CN:H_2O, 5:35:60	1.0	CH_3CN:H_2O, 40:60

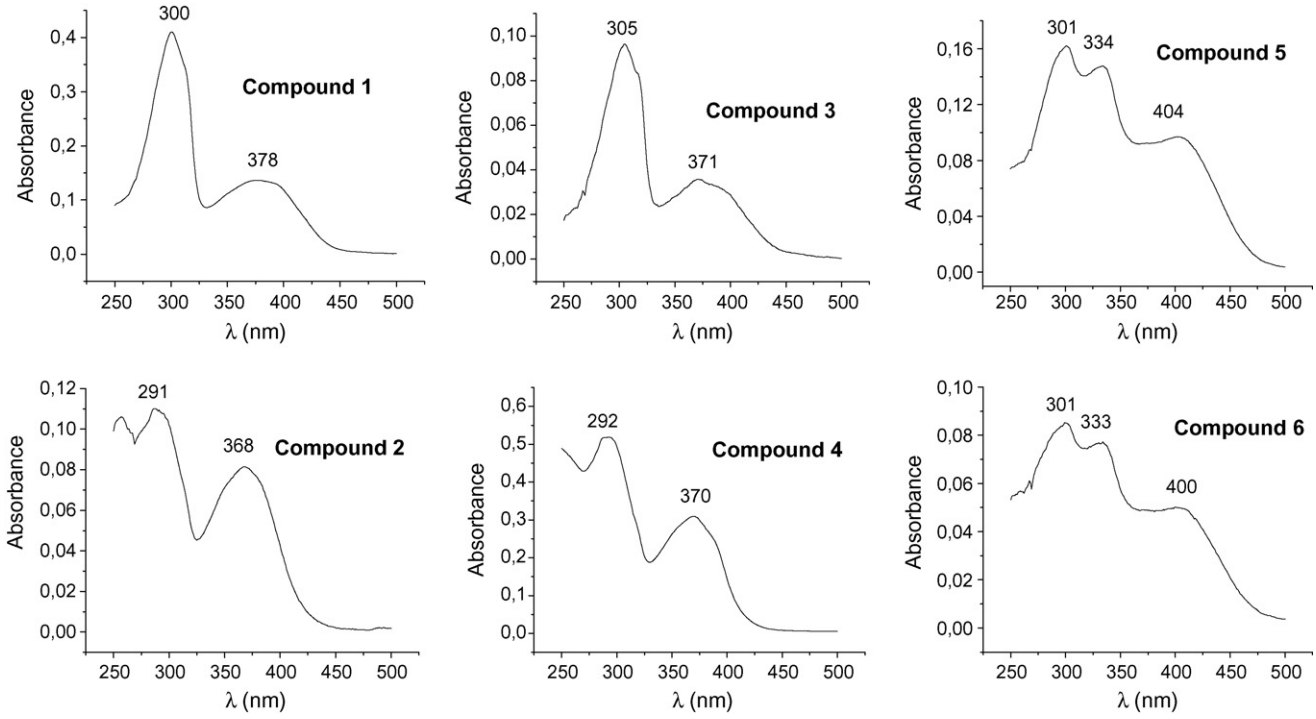


Fig. 3. UV spectra for the different analyzed analytes (MeOH:CH_3CN, 50:50). Top: *E*-isomers **1**, **3**, and **5**. Up: *Z*-isomers **2**, **4**, and **6**.

were treated by linear least square regression analysis. Calibration curves were obtained each day substances were analyzed.

2.4.3. Accuracy and precision

Accuracy and precision of the method were determined for the compounds by analyzing standard samples at three concentrations of studied compounds. The method precision was established by injecting six standard samples at each concentration level for the intra-day precision and on 3 days for the inter-day precision. Precision was expressed by the percentage of relative standard deviation (%rsd) of the analyte peaks. Accuracy was established by evaluating the relative bias between the actual concentration value and the mean value obtained from the calibration curve.

2.4.4. Specificity

Specificity of the method, for each of the studied compound, was determined by analyzing a sample containing a mixture of the four reaction end-products, namely the stereosiomers and the corresponding deoxygenated derivatives.

2.4.5. Limit of detection

The limit of detection (lod) for each studied compound was determined as the concentration that resulted in a signal to noise ratio of 3:1 (S/N = 3) [16,17].

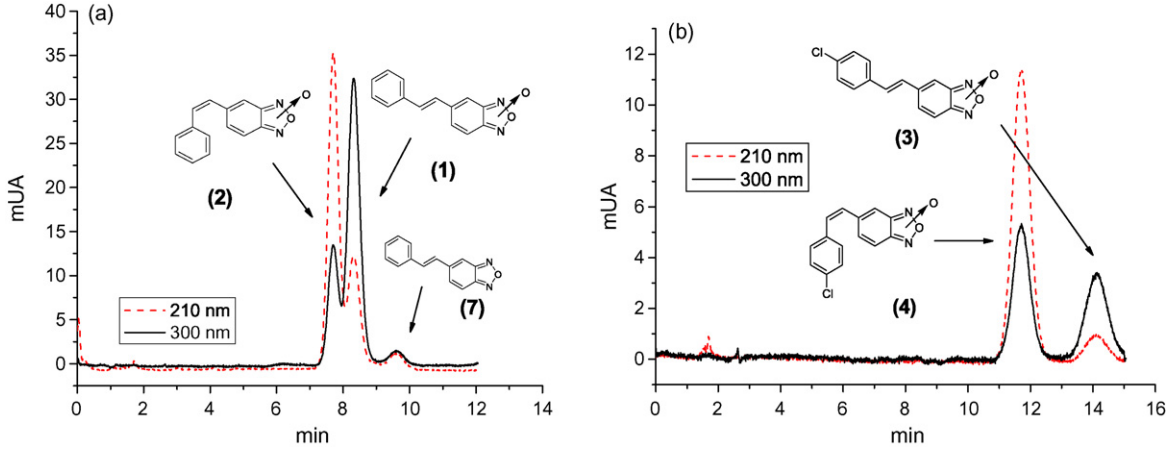


Fig. 4. HPLC profile of the synthetic derivatives using different wavelength (210 and 300 nm) as UV-detection. (a) Scale-up batch in the production of derivatives **1** and **2** (mixture of both isomers and secondary products). (b) Scale-up in the production of derivatives **3** and **4** after chromatographic isolation. Run conditions: reverse-phase Supelco LC-18 column, isocratic elution with mobile phase of acetonitrile:water, 40:60 (v/v), column temperature 27 °C, injection volume 10 mL, flow rate 1.0 mL min^{-1}.

Table 2
Chromatographic parameters of the selected conditions and molar absortibity, at 300 nm, of the studied compounds

Compound	RT (min)[a]	Rs[b]	Column plates	lod (μM)[c]	loq (μM)[d]	ε[e]
1	13.86[f]	1.67	3222	0.6	1.8	26037[g]
2	12.30[f]			3.8	12.6	10780[g]
3	14.52[h]	1.88	1777	1.4	4.6	39313[j]
4	12.00[h]			1.4	4.5	7807[j]
5	10.53[i]	1.97	2138	7.7	25.6	4965[g]
6	8.84[i]			7.2	24.0	13706[g]

[a] RT: retention time, HPLC conditions according to Table 1.
[b] Rs: resolution.
[c] lod: limit of detection.
[d] loq: limit of quantification.
[e] ε: molar absortibity at 300 nm (L mol^{-1} cm^{-1}).
[f] t_o = 2.29 min.
[g] In MeOH:H_2O, 50:50.
[h] t_o = 1.71 min.
[i] t_o = 2.73 min.
[j] In CH_3CN:H_2O, 40:60.

2.4.6. Limit of quantification

The limit of quantification (loq) for each studied compound was determined as the concentration that resulted in a signal to noise ratio of 10:1 (S/N = 10), provided that both accuracy and precision were lower than 15% [18].

2.4.7. Robustness

The robustness of the method was evaluated by analyzing the system suitability standard and evaluating system suitability parameter data after varying, individually, the HPLC pump flow rate (±5%), auto-sampler injector volume (±20%) and column compartment temperature (±2 °C).

3. Results and discussion

3.1. Optimization of HPLC methods

The studied compounds, desired and secondary products, are small apolar neutral molecules. Derivatives **1–6** (Fig. 1) exist in solution and at room temperature as ring-chain tautomers (Fig. 5) [19–21]. These equilibriums are very fast and they are chromatographically undetectable in the assayed conditions. The compounds could be studied on reverse-phase HPLC columns however silica column was also studied. Nevertheless, the high apolar characteristics of the studied compounds have not allowed us to obtain adequate RT and Rs (being in all the studied cases lower than 1.2) in silica and in the studied mobile phase (mixture of petroleum ether and ethyl acetate). The optimization goal was to develop a simple chromatographic method for each geometric isomer and its deoxygenated derivatives with the most efficient analysis time. Typically, method development focuses on implementing small solvent changes to optimize selectivity and enhance resolution. An overview of our method optimization which includes the selection and test results of different mobile phases is listed in Table 3. In summary, C-18 phase and the binary or ternary solvent systems shown in Table 1 were selected as chromatographic conditions.

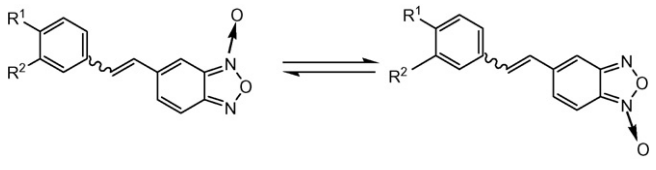


Fig. 5. Tautomeric equilibrium of studied benzofuroxans.

3.2. Method validation

The following method validation characteristics were addressed for each studied compound: linearity, accuracy, precision, specificity, limit of detection and quantification and robustness. Therefore, the method validation for the studied compounds meets the requirements for USP Category I [22].

Table 3
Some selected conditions in the method development using as bonded phase C-18

Compound	Mobile phase	Flow rate (mL min^{-1})	RT (min)	Rs
1	MeOH:H_2O, 50:50	0.8	15.21	1.27
2			13.53	
1	MeOH:H_2O, 50:50	1.0	12.32	1.31
2			10.90	
1	MeOH:H_2O, 45:55	1.0	19.12	1.45
2			16.46	
1	MeOH:CH_3CN:H_2O, 40:10:50	0.8	10.32	1.48
2			9.37	
1	MeOH:CH_3CN:H_2O, 40:10:50	1.0	8.34	1.53
2			7.55	
1	MeOH:CH_3CN:H_2O, 45:5:50	1.0	11.39	1.62
2			9.99	
1	MeOH:CH_3CN:H_2O, 35:10:55	1.0	15.93	1.63
2			13.72	
3	MeOH:CH_3CN:H_2O, 35:10:55	1.0	14.08	1.82
4			11.67	
5	CH_3CN:H_2O, 40:60	1.0	8.10	1.37
6			7.25	

Table 4
Parameters and linearity data of studied compounds calibration curves

Compound	Analytical range (μM)	Calibrators	Slope	*y*-intercept	r^2
1	1.8–80.9	6	9.0	0.8	0.9971
2	12.6–991.6	6	37.0	−8.0	0.9990
3	4.6–86.8	6	27.4	0.3	0.9992
4	16.8–593.9	6	27.5	1.5	0.9994
5	25.6–156.0	6	18.2	0.7	0.9960
6	24.0–601.8	6	40.7	2.2	0.9998

3.2.1. Linearity

The results, summarized in Table 4, show excellent correlations between analyte peak area and concentration of the studied compounds with $r^2 \geq 0.9960$ ($n = 18$).

3.2.2. Accuracy and precision

Table 5 summarizes both the accuracy and precision of the method. Accuracy is stated as the percentage of the actual concentration value (acv). Non-significant differences were observed between inter-day and intra-day results for both %acv (Student *t*-test for the difference of means) and %rsd (Fisher *F*-test for the ratio of variances). Then, results included in Table 5 are the worst values obtained for each parameter.

3.2.3. Specificity

Samples containing mixtures of each stereoisomer, *E* and *Z* geometric isomers, were analyzed (Table 2). Additionally, samples containing mixtures of each geometric isomer and the corresponding deoxygenated analogue were analyzed (Table 6). No co-eluting substances were observed, at least spectrophotometrically, in any of the samples. In addition, resolution between the analyzed products was always greater than 1.67. Due to the absence of any co-elution in any of the samples we determined this method to be specific for the desired benzofuroxans.

Table 5
Accuracy and precision ($n = 6$)

Compound	Concentration (μM)	Accuracy (%acv[a])	Precision (%rsd[b])
	4.6	97.8	4.8
1	46.2	101.9	3.7
	80.9	93.3	4.9
	24.8	98.4	2.5
2	247.9	101.5	4.1
	867.7	102.6	5.5
	4.9	102.0	9.7
3	38.9	100.5	2.6
	88.3	100.7	2.7
	34.0	102.6	2.4
4	252.8	100.6	1.2
	592.4	100.8	2.2
	33.9	99.1	6.9
5	42.5	94.4	4.9
	156.0	99.9	3.2
	35.4	92.7	13.0
6	194.7	99.7	2.5

[a] %acv: percentage of the actual concentration value.
[b] %rsd: percentage of relative standard deviation.

3.2.4. Limit of detection and quantification

The lod and loq of these methods were obtained for all the studied compounds (Table 2).

3.2.5. Robustness

To ensure the insensitivity of the HPLC method to minor changes in the experimental conditions it is important to demonstrate robustness of the method. None of the alterations, HPLC pump flow rate, auto-sampler injector volume and column compartment temperature, caused a significant change in resolution between the studied compounds and the impurities, peak area, peak width or theoretical plates.

3.3. Analysis of the drugs scale-up procedures

The validated methods were used in the analysis of each of the phenylethenylbenzofuroxans production (Scheme 1). These included the study of the different stages in the synthetic procedure for each of the desired compound (representative example is shown in Fig. 6, production of desired compound **4**) or the study of the quality of the final products from different synthetic batches. Representative results for **3** and **4** production in three different batches, after purification procedures (end-products) are summarized in Table 7.

Table 6
Retention time of benzofuroxans and the corresponding deoxygenated analogues

Compound	Mobile phase	Flow rate (mL min^{-1})	RT (min)[a]
1	MeOH:CH_3CN:H_2O, 45:5:50	0.8	13.86[b]
7			16.04[b]
2	MeOH:CH_3CN:H_2O, 45:5:50	0.8	12.30[b]
8			13.90[b]
3	CH_3CN:H_2O, 40:60	1.0	15.04[c]
9			17.81[c]
4	CH_3CN:H_2O, 40:60	1.0	12.13[c]
10			14.51[c]
5	MeOH:CH_3CN:H_2O, 5:35:60	1.0	10.53[d]
11			12.09[d]
6	MeOH:CH_3CN:H_2O, 5:35:60	1.0	8.84[d]
12			10.14[d]

[a] RT: retention time.
[b] $t_o = 2.29$ min.
[c] $t_o = 1.71$ min.
[d] $t_o = 2.73$ min.

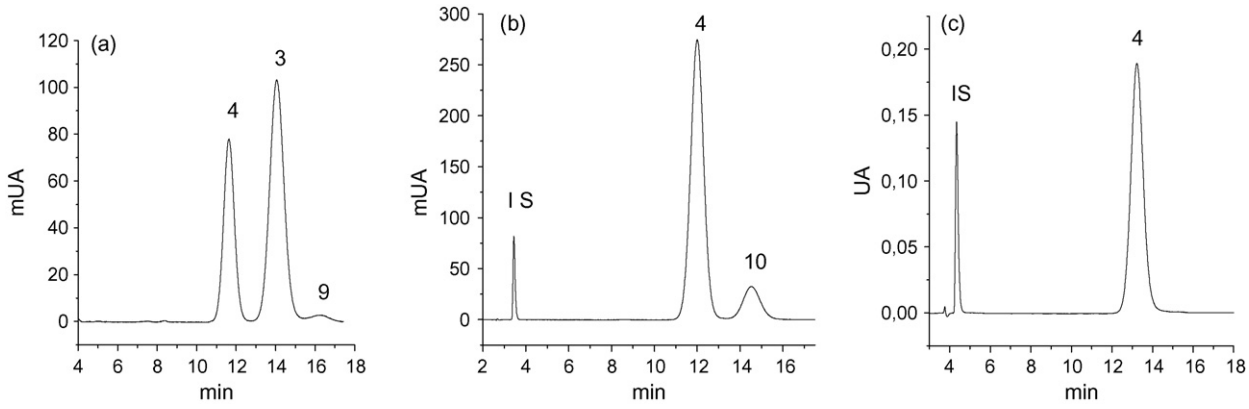


Fig. 6. Examples of HPLC analysis of different stages in the synthesis of 5*Z*-[2-(chlorophenyl)ethenyl]benzofuroxan, **4**. (a) Mixture of scale-up reaction (Scheme 1). Conditions according to Table 1, t_o = 0.70 min. HPLC analysis and NMR spectroscopy confirm the presence of **3** as secondary product and **9** and **10** as impurities of desired compound **4**. (b) Fraction corresponding to compound **4** after isolation by chromatographic column (Scheme 1). Conditions according to Table 1, RT_{IS} = 3.38, t_o = 1.60 min. This fraction was contaminated with product **10** according to HPLC analysis. (c) Fraction corresponding to compound **4** after purification by crystallization from petroleum ether:EtOAc, 90:10 (Scheme 1). Conditions according to Table 1, t_o = 2.55 min.

Table 7
HPLC results of different synthetic batches in the **3** and **4** productions (end scale-up products after chromatographic process and purification by crystallization)

Batch	Purity[a]	
	3	**4**
1	99.9	99.6
2	99.8	99.9
3	99.7	99.8

[a] Purity refers to each sample of studied compound after each individual isolation and purification processes. Each derivative was isolated and purified according to reference [12] (Scheme 1).

4. Conclusion

Simple and efficient reverse-phase HPLC methods for analyzing 5-phenylethenylbenzofuroxans were found to be accurate, precise, and linear across the analytical range. The methods were specific for the determination of the desired products and its secondary products. The methods could be used to assess the quality of the scale-up reaction mixtures or the final scale-up products.

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