Determinación por Cromatografía Líquida de Alta Resolución de 5-bromo-4-cloro-3-indolil-β -D-galactopiranósido (Xgal) y los intermediarios de su síntesis

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Palabras clave: 5-bromo-4-cloro-3-indolil-β-D-galactopiranósido, Xgal, HPLC, validación, RP-18.
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RESUMEN. En microbiología, los indicadores de microorganismos son muy utilizados en la determinación de la calidad de diferentes productos entre los cuales se destacan el agua y los alimentos, donde los coliformes, especialmente de *E. coli* se han identificado. Entre estos indicadores se destacan los derivados de indoles, que tienen la propiedad de comportarse como un sustrato cromogénico. Uno de estos es el 5-bromo-4-cloro-3-indolil-β-D-galactopiranósido (Xgal) y se emplea como componente de los medios de diagnóstico microbiológicos. Se obtiene mediante la síntesis química y en presencia de coliformes productores de la enzima β-galactosidasa muestra una coloración azul índigo, por tal motivo es empleado en la identificación y cuantificación de estos microorganismos. En este trabajo, se reporta el desarrollo y validación de una técnica analítica por HPLC que permite evaluar la calidad del producto final y de los intermediarios que se obtienen en cada etapa de síntesis. Con el empleo de una columna de fase reversa y de una fase móvil compuesta por una disolución estabilizadora de fosfato pH 3 y acetonitrilo, así como la utilización de un gradiente de elución, se logró separar el 5-bromo-4-cloro -3-indolil-β-D-galactopiranósido de cada uno de sus intermedios de la síntesis. El método desarrollado tiene un límite de detección del 0,2 al 0,5 % y un límite de cuantificación del 1,0 al 2,0 %, el cual es aceptable si se tiene en cuenta que el Xgal se utiliza como un reactivo de diagnóstico. El método desarrollado es sencillo, lineal, selectivo, preciso y exacto, por lo que puede emplearse en el control de calidad de cada uno de los intermediarios que se obtienen en cada etapa de síntesis y del producto final.

ABSTRACTS. In microbiology, microorganisms markers have been found to be useful in the quality control of different products such as water and food, in which the presence of coliforms, especially *E. coli* has been identified. Several methods for detection and quantification of enterobacteria have been developed by using chromogenic substrates. Among them, indole derivatives such as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal), have come out. This indole derivative is widely employed as a constituent of microbiological diagnostic media because in the presence of coliforms (β-galactosidase producers), it forms a blue-indigo color. In this paper, the development and the validation, of an HPLC method for the quality control of Xgal in the presence of its intermediates obtained in each step of the synthesis is reported. These compounds were separated by using a RP-18 column and gradient elution with a mobile phase consisting of triethylammonium phosphate solution at pH 3(A) and acetonitrile (B). The detection and quantitation limits of the method was between 0.2 – 0.5 % and 1.0 – 2.0 % respectively, which is acceptable if we consider that Xgal is not used as a drug. The proposed method is simple, selective, linear, precise and accurate and it can be used for the quality control of each of the intermediates and the final product, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

INTRODUCIÓN

In microbiology, microorganisms markers have been found to be useful in the quality control of different products such as water and food, in which the presence of coliforms, especially *E.Coli* has been successfully identified.[1-7]

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There are numerous methods, with different degrees of complexity and accuracy, to identify and determine coliforms (E.Coli), in presence of some other bacteria. Some of these methods only evaluate the presence or absence of microorganisms, other methods allow to quantify them. Detection and quantification methods for enterobacteria have been developed by using chromogenic substrates. The use of more sensitive methods led to improve the accuracy and rapidity of the detection, with the principal advantage of permitting the simultaneous detection of different bacteria by means of the selection and the adequate combination of various substrates in the same diagnostic medium.[7]

Among them, indole derivatives such as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal, **I**), have come out. This indole derivative is widely employed as a constituent of microbiological diagnostic media because in presence of coliforms (β-galactosidase producers), it forms a blue-indigo color.[7] This property allows determining the contamination degree due to the presence of microorganisms pathogens with the minimum diffusion in the diagnostic medium employed.

For this reason, Xgal is also employed particularly in molecular biology applications for detecting the β-galactosidase enzymatic activity, usually used as reporter gen in the cloning process,[8-10] for detecting the presence of foreign DNA in the plasmid DNA lacZ region, leading to the loss of the β-galactosidase activity.[11] Other applications include the immunoassays, where the β-galactosidase is specifically link to an antibody, and also for detecting the micro metastasis during the progress of a tumor.[12-15]

In the Center of Pharmaceutical Chemistry a novel route of synthesis for Xgal was developed by modification of Roth and Ferguson procedure.[7] The analytical information of Xgal and its intermediates from synthesis is restricted. However, to determine indoles and their derivatives some HPLC methods have been reported.[16-23] Generally, those methods use a reverse phase column, UV detection and mixtures of acetonitrile-water or methanol-water in different ratios and pH as mobile phases. In function of the sample complexity, separation takes place using isocratic programs, gradient elutions and organic additives. To regulate the pH, is frecuent the use of phosphate, formiate, citrate and acetate buffers, and for obtaining narrow peaks on the chromatograms triethyl amine is employed as organic additive.[24-26] These methods quantify indole derivatives in different matrixes but any of them has been used to determine Xgal and its intermediates from synthesis up to the present. For this reason, a new method for the quantitative determination of these products was required. That is why, the purpose of this paper was to develop a method to carry out the quality control of Xgal and each intermediate obtained from the synthesis process (Fig. 1).

MATERIALS AND METHODS

Apparatus

The chromatographic separation was performed with a Merck-Hitachi apparatus constituted by a gradient pump L-7100 with a Rheodyne 7125 injector provided with a 20 μL loop and a UV-Vis detector L-7400. The acquisition and data analysis was performed in a personal computer equipped with the chromatographic software Biochrom Ver. 1.2 (Center of Genetic Engineering and Biotechnology, Cuba).

Reagents

The reagents employed in this work were supplied by Merck (Germany), all of them were of analytical grade and the solvents utilized were of HPLC quality. Reference substance of Xgal (I, purity 99 %) was supplied by Fluka and each of the related compounds from synthesis was obtained from the Center of Pharmaceutical Chemistry, Havana, Cuba. The identities and purity of these compounds were confirmed by thin layer chromatography and NMR analysis.[27]

Preparation of solutions

Samples were prepared in methanol to a concentration of 1 mg/mL and diluted up to working concentrations with mobile phase A. The concentrations for the determination of I, II III, IV and V were 0.21, 0.19, 0.23, 0.22 and 0.28 mg/mL respectively. The samples were stored at refrigerated conditions (2-8 °C).

Experimental part

To carry out the separation, a reverse phase column (18-RP), 125 x 4 mm i.d., with a particle size of 5 μm (LiChrospher 100, Merck) was used. Detection took place at 254 nm, a wavelength at which all theses organic compound adequately absorb; the flow rate was 1.0 mL/min and the injection volume was 20 μL . As mobile phases, a solution containing 0.05 mol/L sodium dihydrogen phosphate and 0.03 mol/L triethylamine, adjusted to pH 3 with 85 % orthophosphoric acid (A) and acetonitrile (B) were used. The samples were filtered through a nylon membrane filters of 0,45 μm (Sartorius, Germany) before the chromatographic analysis.

Validation parameters

Selectivity, linearity, precision, and accuracy were determined according to reported procedures.[28-30] All statistical calculations were made by means of Microsoft Excel Windows.

Two ranges of concentration were examined in the linearity study. The higher one considered each compound as the main product of its synthesis and the lower one considered compounds *II*, *III*, *IV* and *V* as impurities of *I*. The concentration ranges were 0.15 - 0.41 mg/mL for compound I, 0.15 – 0.39 and 0.003 – 0.02 mg/mL for *II*, 0.17 - 0.45 and 0.004 -0.02 mg/mL for *III*, 0.16 – 0.43 and 0.004 – 0.02 mg/mL for *IV* and 0.21 – 0.55 and 0.002 – 0.01 mg/mL for *V*.

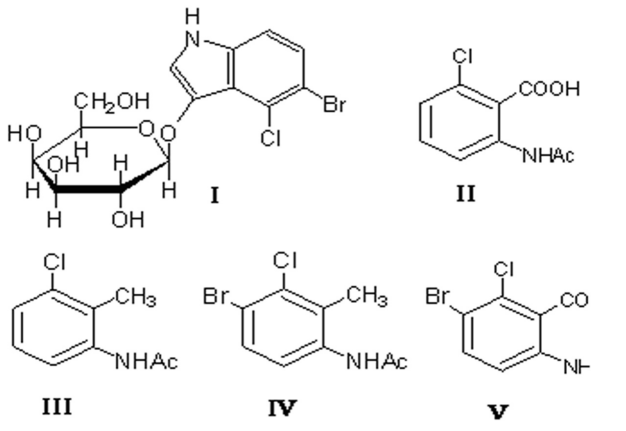

Fig. 1. *Chemical structures of 5-bromo-4-chloro-3-indolyl-β–D-galactopyranoside (I) and its related compounds from synthesis: 2-N-acetyl-6-chorobenzoic acid (II), 3-chloro-2 methyl-acetanilide (III), 4-bromo-3-chloro-2-methyl-acetanilide (IV) and 2-N-acetyl-5-bromo-6-chlorobenzoic acid (V).*

The precision study was carried out on two different days by two different analysts.

To do the accuracy study, three samples of known concentration within the linear range were prepared for each compound and the determination was achieved. Then, a regression analysis was performed, comparing calculated concentration versus theoretical concentration, so the linear correlation coefficient and the confidence limits for the slope and the intercept for each compound were calculated. A homogeneity analysis for variances was done to prove the influence or not of concentration in the results, for this purpose the Cochran test is applied, taking into consideration three concentration levels (0.1, 0.2 and 0.3 mg/mL). On the other hand, the limit of detection (LOD, at a signal-to-noise ratio, s/n ≥ 3) and the limit of quantification (LOQ, S/N ≥10) were determined. A stability study of the samples prepared in mobile phase A at pH 3 and stored under refrigerated conditions was made during two different days.

RESULTS AND DISCUSSION

Method development

The first choice to perform the separation of Xgal and its intermediates was the use of a mobile phase containing 70 % of A and 30 % of B (Fig. 2). By using this condition, peaks corresponding to compounds *II* and *V* are eluted very close to the solvent peak and there is no baseline separation. On the contrary, peaks corresponding to compounds *I*, *III* and *IV* were separated successfully. By decreasing the mobile phase B up to 25 %, baseline separation between peaks *II* and *V* was achieved, but peak *II* was still close to the solvent peak. Also an increment of the retention times and the peak width of *III* and *IV* were observed. Therefore it was decided to carry out gradient elution. The chromatograms performed by the use of two elution gradients were obtained with complete separation of all the components up to baseline (Fig. 3), peak *II* was well separated from the solvent peak and the peak *IV* remains sufficiently narrow so that it can be quantified without problems.

The elution order of compounds *II, V, III* and *IV* was as expected. The presence of the carboxyl group attached to the aromatic ring for compounds *II* and *V* increases their polarity in respect to compounds *III* and *IV* that carry a methyl group in their structure and so *II* and *V* are eluted first. On the other hand, the presence of the bromine atom in compounds *IV* and *V* leads to a decrease of the molecule polarity with respect to compounds *III* and *II* respectively; hence *IV* and *V* are eluted later. The position of compound **I** in the chromatogram indicates that its polarity is intermediate.

In accordance with these results, it can be concluded that it is possible to use any of the elution gradients proposed to carry out the separation of *I* from its related compounds. Gradient II was selected to be used in further work, because it was somewhat faster.

Validation

All the compounds analyzed were well resolved (Table 1). The resolution and capacity factors were superior to 1.5 and 2.0 respectively and the selectivity factor (α) is different to 1. Then, the method is considered to be selective.[18-19]

The linearity study of the method (Table 2) showed that there were no problems in the concentration range studied. For each compound, correlation coefficients superior to 0.998 and non significant intercepts were found.

The precision was verified at 0.2 mg/mL (67 %) and 0.006 mg/mL (3 %) for each compound analysed (Table 3).

The RSD calculations were carried out on the area values obtained by integration. As it is observed, for the concentration of 100 %, the relative standard deviation for each analyst (RSD_1 and RSD_2) and the global relative standard deviation are lower than those established as limits, 2 and 3 % respectively. For 3 % the RSD_1 and RSD_2 were higher, as expected. During this study no statistical differences for the peak areas of each compound were found, indicating that the samples are stable under the storage conditions at least by two days of analysis.

For this reason, the method is considered to be repeatable and precise.[29-30] The accuracy study illustrates that

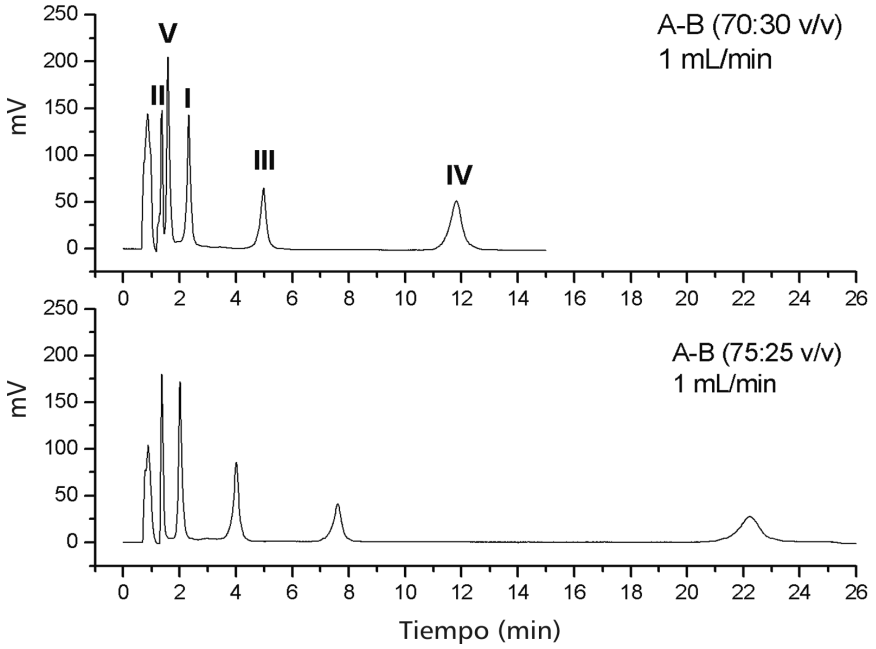

Fig. 2. *Chromatograms of Xgal and its intermediates obtained by using two different mobile phase compositions with isocratic elution.*

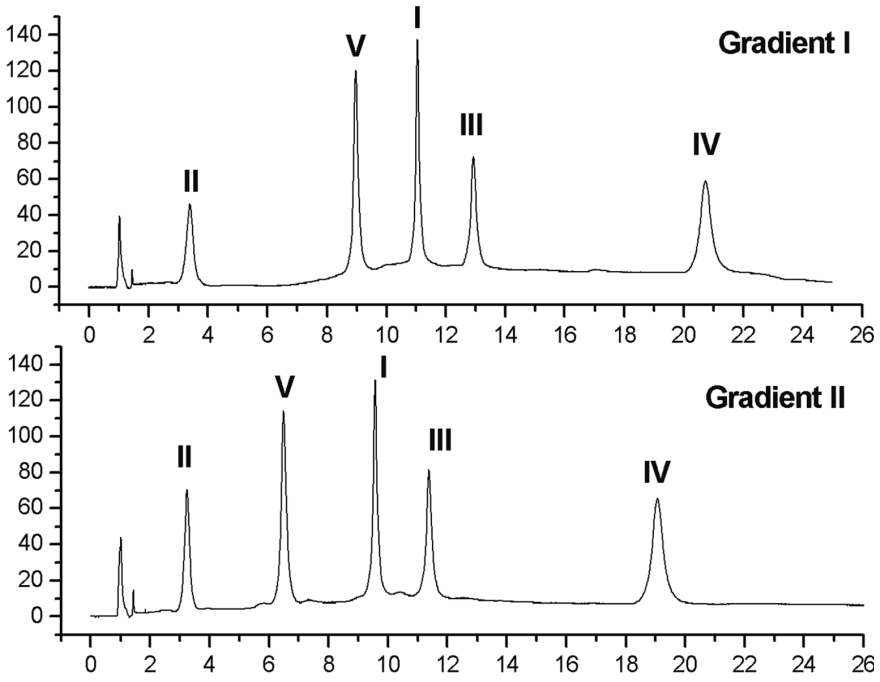

Fig. 3. *Chromatograms of Xgal and its intermediates obtained by using two different elution gradients: gradient I, 4 min with 10 %B, 4 min with a linear gradient from 10 %B to 30 %B and 12 min with 30 %B; gradient II, 8 min with a linear gradient from 10 %B to 30 %B and 14 min with 30 %B.*

the confidence limits of intercept and slope values for each compound include 1 and 0 respectively (Table 4), the linear regression coefficients are higher than 0.998 in each case, therefore the method is able to determine exactly the content of each compound present in the samples. The results of the Cochran test (G) for the three concentration levels (0.1, 0.2 and 0.3 mg/mL), show that the results are not influenced by the concentration. For compounds *II, III, IV* and *V* the G values were 0.453, 0.857, 0.504 and 0,764 respectively, all of them lower than the critical value G (0.05; 3; 2) = 0.871.[31]

Table 1. Resolution, capacity factor and selectivity values calculated for 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (I) and its related compounds from synthesis.

Compound	k´		Rs	α
II	2,3	II - V	2,3	2,5
V	5,7	V - I	2,4	1,6
I	9,0	I - III	1,5	1,2
III	10,9	III - IV	3,8	1,7
IV	18,8			

k' Capacity factor. Rs Resolution. α Selectivity factor. All of them calculated by using chromatogram obtained with gradient II.

The ranges of detection and quantification limits were 0.2 – 0.5 % and 1.0 – 2.0 % (0.3 mg/mL = 100 %) (Table 5). A chromatogram of the Xgal spiked with 0.2 % of each intermediate demonstrates that the method is capable to detect the intermediates present in the sample (Fig. 4). In the stability study of the samples no statistical differences were found between the peaks areas obtained during two different days. These results are acceptable if it is considered that Xgal is not used as a drug.[29]

CONCLUSIONS

By the use of a mobile phase A constituted by a triethylammonium phosphate buffer pH 3, acetonitrile as mobile phase B and a gradient elution, the separation of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and its related compounds from

Table 2. Linear regression parameters for Xgal and its related compounds from synthesis.

Parameters values of the linear regression model. y = b x + a	I	II	III	IV	V
Intercept (a)	–0.18	–3.39	–2.19	-3.10	–7.26
Slope (b)	204.83	422.25	231.75	414.62	483.34
r	0.998 6	0.998 7	0.999 1	0.998 4	0.998 5
$t_{exp.}$ (intercept)	–0.126	–1.266	–1.559	–0.951	–1.557

r Linear correlation coefficient. Texp. (intercept) T Student calculated for the intercept. T(0.05; 4) = 2.776.[31] y Peak area. x Concentration (mg/mL).

Table 3. Results of the precision study.

Considering each compound as the main product (100 %)		I	II	III	IV	V
Intraday repeatability (n = 6)						
Analyst 1	RSD_1	1.2	0.8	0.9	0.8	1.2
Intraday repeatability (n = 6)						
Analyst 2	RSD_2	1.7	1.7	1.4	1.6	1.2
Interday repeatability (n = 12)	$RSD_{(global)}$	1.9	2.0	2.0	1.9	2.0
Considering each compound as impurity (3 %)						
Intraday repeatability (n = 6)						
Analyst 1	RSD_1	—	6.3	12.4	27.9	23.3
Intraday repeatability (n = 6)						
Analyst 2	RSD_2	—	11.4	18.2	27.1	27.8
Interday repeatability (n = 12)	$RSD_{(global)}$	—	13.7	18.3	23.1	23.4

RSD Relative standard deviation expressed in percent (%).

Table 4. Results of the accuracy study.

Regression parameters	II	III	IV	V
Intercept (a)	–0.001	–0.001	–0.002	0.000
Intercept error	0.004	0.002	0.004	0.004
Texp. (a)	–0.423	–0.862	–0.966	–0.088
Slope (b)	0.99	1.00	1.01	1.01
Slope error	0.02	0.01	0.02	0.02
Texp. (b)	125.02	218.62	132.36	148.58
r	0.999	0.999	0.999	0.999

RSD Relative standard deviation expressed in percent (%).

Table 5. Detection (LOD) and quantification (LOQ) limits calculated for each compound.

Compound	LOD (%) S/N = 3	LOQ (%) S/N = 10
II	0.2	1.0
III	0.5	2.0
IV	0.5	2.0
V	0.2	1.0

S/N Signal/noise ratio.

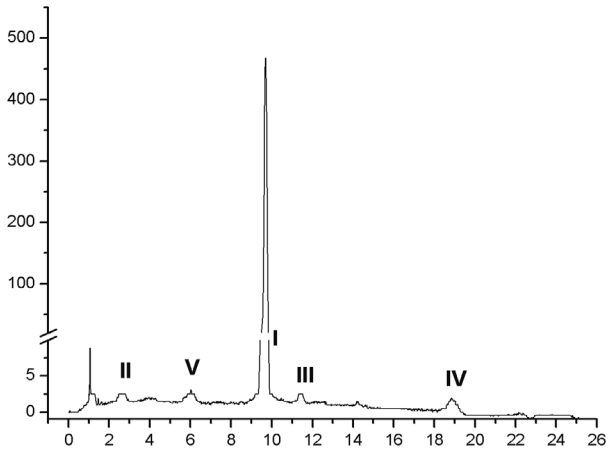

Fig. 4. *Chromatogram of Xgal in presence of 0.2 % of each impurity by using the elution gradient II.*

synthesis was achieved. The method was validated to be selective, linear, precise and accurate and it can be used to evaluate the quality of each of the intermediates and of the final product, 5-bromo-4 -chloro-3-indolyl-β-D-galactopyranoside. These products can be detected in the detection limits range of 0.2 – 0.5 % and they can be quantified in the quantification limits range of 1.0 – 2.0 %, which is acceptable if it is considered that I is not used as a drug.

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