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Optimization and Modelling of The Analysis of Pleuromutilin Antibiotic Extracted From Biomass

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An isocratic and simple high performance liquid chromatographic method was developed for the direct resolution of pleuromutilin antibiotic extracted from biomass. The method uses Beckman coulter, ODS, C_8, 450 × 4,6 mm I.D. Several mobile phases, flow rates and temperatures were checked in order to optimize the resolution. UV detection at 212 nm was considered. The method proved to be able to resolve pleuromutilin antibiotic. The best base line separation was carried out using mobile phase consisting of acetonitrile and buffer in ratio 60/40, column of C_8 at 35 °C. The optimized method proved an important improvement of the retention time, which decreased from a value of 10.8 min found in the available literature to only 2.5 min.

Key Words: Antibiotics, Biomass, Detection, Liquid chromatography, Modelling, Pleuromutilin, *Pleurotus mutilis*.

INTRODUCTION

Pleuromutilin (Fig. 1) was first discovered in the basidiomycete, *Pleurotus mutilus*, which is now named *Clitopilus scyphoides*[1-3]. The diterpene antibiotic pleuromutilin was first removed by Kavanagh in 1951. The compound is active against a variety of drug-resistant Gram-positive bacteria and mycoplasmas and is an inhibitor of bacterial protein synthesis[4,5].

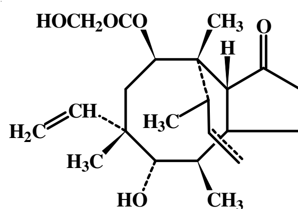

Fig. 1. Structure of pleuromutilin

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Birch[6] initiated and extensively studied the chemical and structural features of pleuromutilin. He determined the complete structure of pleuromutilin, a unique fused 5-6-8 tricyclic skeleton with eight asymmetric centers, three of which are quaternary. The drug is soluble in water and readily absorbed. It is therefore amongst the few antibiotics that can be easily administered to animals. So far, pleuromutilins are only recommended in veterinary practice and most frequently for swine dysentery remedy. However, the increasing number of pathogens resistant to common antibiotics raised a new interest in pleuromutilins and their derivatives, which may be suitable for human therapy[7-11].

Table-1 shows the physico-chemical characteristics of Pleuomutilin antibiotic[12] microbial as well as chemical methods were described for pleuromutilin determination. These methods lack specificity and ability to identify the composition of this multi-component drug. The application of liquid chromatography (LC) has increasingly demonstrated the complex nature of this antibiotic. Rodriguez and co-workers[13] separated the semi-synthetic derivative of the naturally occurring antibiotic pleuromutilin produced by the fungus *Pleurotus mutilis* on ion-exchange columns and other polar stationary phases, at pH 7.0 and UV detection at a wavelength of 208 nm[14].

TABLE-1
PHYSICO-CHEMICAL TESTS OF PLEUROMUTILIN

Aspect	Specification
Identification	by HPLC
Loss with the desiccation	< 8.0 %
Relative total substances by HPLC	< 8.0 %
Impurities	< 6.0 %
Proportioning	> 92.0 %
Melting point	163-165 ºC

A method for the analysis of several macrolide and ionophore antibiotics as well as tiamulin in liquid manure was developed by Schlüsener and co-workers[15]. Reverse-phase liquid chromatography and atmospheric pressure chemical ionization tandem mass spectrometry was used for detection. High-performance liquid chromatographic (HPLC) separation of the antibiotic was achieved in 35 min. The analytes were removed with ethyl acetate and the extracts were cleaned up by solid-phase extraction on a diol SPE cartridge[16].

The high retention times reported in the literature[17] can negatively affect on the development of pleuromutilin determination by HPLC. Enhancing the efficiency of the retention time of pleuromutilin was therefore the purpose of this paper to achieve an acceptable retention time for routine determination. In this aim, the experimental conditions for analysis were optimized, namely the composition of the mobile phase, the flow rate and the temperature, since this optimization has not been previously reported in the available literature.

EXPERIMENTAL

Pleuromutilin extracting process

Broth reception: At the end of culture, namely after 9-10 days, for a title in the range 5000 to 8000 µg/g, broth dilution was carried out with softened water and then maintained under agitation at a temperature of 15-20 °C during the time-storage, which should not exceed 36 h.

Rotary filter preparation: A dicalite suspension (3 % w/v) was prepared and added into the tank of the rotary filter. At a maximum speed of rotation (1 rev/min) of the rotary filter, the dicalite suspension was filtered, inducing an empty in the cylinder and hence a 2-3 cm layer was formed on the filter. The operation was repeated 3 times to complete the retentate layer.

During the filtration, the scraper of the biomass was kept in a position allowing to reduce the thickness of the layer of dicalite. It can be noted that in case of a too thin layer the preparation of a second layer may be considered.

Broth filtration on the rotary filter: The filtration of the broth was carried out at the minimal speed rotation (0.5 rev/min). The volume of the broth in the rotary filter tank was maintained constant during the procedure. After filtration of 200 cm^3 of broth, the titration of mother liquors was considered and should be in the range 800 to 1000 µg/g.

The biomass retained on the filter was scratched, then transported on a treadmill and harvested in a storage tank in view of extraction. It represented 25 % of the total fermentation broth (Fig. 2).

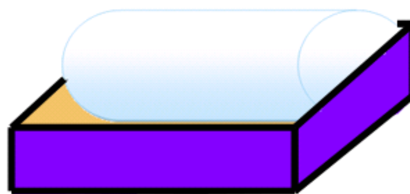


Fig. 2. Diagram of the rotary filter

Methanol extraction: Methanol induces a complete dissolution of pleuromutilin and hence was used for its extraction, which was carried out as follows: (a) A quantity of methanol corresponding to 2.5 times the volume of biomass was added to completely cover the biomass; (b) The obtained solution was maintained under continuous stirring, at a temperature in the range 6 to 8 °C by cooling the tank to avoid the loss of methanol by evaporation; (c) After 8 h of agitation, a sample was taken to estimate the rate of extraction by HPLC analysis. If the title was between 4000 and 7000 µg/g, a filtration on a filter-press can be carried out. Instead, the stirring time should be increased by 2 h to obtain the standard yield of extraction. The methanol containing the extracted pleuromutilin was then stored.

Crystallization: The crystallization consists of pleuromutilin crystal formation by correcting the pH, as described in the following procedure: (a) The reactor was loaded with 100 cm^3 of the filtrate; the initial pH was between 7 and 7.5; (b) The pH was then corrected by the addition of caustic soda solution (NaOH 5 %) until a pH value of 8.3-8.4 and hence the appearance of crystals.

The several solvents were used to measure the solubility of pleuromutilin. The analytical grade solvents purchased from Fluka (Seize, Germany). Acetonitrile used for high performance liquid chromatography (HPLC), as well as water liquid chromatography were purchased from Fisher Scientific (Springfield, NY, USA). For HPLC a Shimadzu series SIL-10ADVP apparatus (Kyoto, Japan) was used for quantitative analysis. The apparatus was equipped with an HPLC pump 10AVP, UV detector, column oven CTO 10AVP, degasser DGU -12A (Kyoto, Japan). All devices were controlled by a computer PC IV (Intel ®, Pentium 4 CPU 280 GHz).

Quantitative HPLC analysis: For quantitative measurements of pleuromutilin antibiotic, a Discovery Supelco C_{18}, 5 µm, 250 × 4.6 mm I.D and Beckman coulter ODS C8, 450 × 4.6 mm I.D. Columns were used. An elution composed of acetonitrile and buffer solution at pH 7.5 was employed with a UV detector at 212 nm. Various flow rates and mobile phases were considered. The mobile phase was composed from acetonitrile and buffer solution tested at the following ratios: 55/45, 60/40, 70/30, 80/20.

RESULTS AND DISCUSSION

Obviously there is no doubt that successful liquid chromatography depends on the right selection of solvent systems. However, as a general rule, the ability of a solvent to remove the solutes is in close relationship with its own capacity to dissolve the solutes. Therefore, solubility of the solutes in various solvents, which is one of their most important physico-chemical properties related to HPLC, should be first analyzed. A series of usual solvents, water, methanol, ethanol, chloroform, *n*-hexane, toluene and ethyl acetate were tested for their efficiency to solubilize pleuromutilin. The results were qualitatively expressed (Table-2).

TABLE-2
SOLUBILITY OF PLEUROMUTILIN IN VARIOUS SOLVENTS

Solvents	Solubility
Water	Practically insoluble
Methanol	Highly soluble
Chloroform	Freely soluble
n-Hexane	Practically insoluble
Toluene	Sparingly soluble
Ethyl acetate	Freely soluble

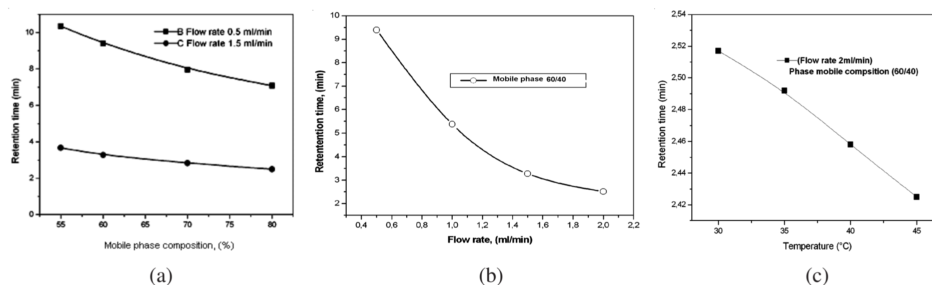
Effect of the mobile phase: The purpose of this study was to select a suitable mobile phase, giving a satisfactory retention time for the chromatographic peaks of

interest, namely yielding to appropriate chromatographic conditions for the detection of pleuromutilin antibiotic. The best limit of detection among the tested mobile phases is sought, leading to sharp symmetrical peaks with satisfactory peak heights. High flow of the mobile phase leads to increasing pressure in the system High speed reduces the time required to separate the components along the stationary phase, as it can be seen at the comparison of two flow rates, 0.5 and 1.5 mL/min (Fig. 3a).

The mobile phase containing a ratio of 60/40 acetonitrile /buffer led to the best resolution.

Effect of the flow rate: The effect of the flow rate on the retention time was examined. As observed, the retention time decreased until less than 3 min for increasing flow rates of the mobile phase (Fig. 3b), without loss in the peak resolution.

Effect of the temperature: The effect of the temperature on the retention time of pleuromutilin antibiotic was investigated in the range 30 to 45 °C. The results showed that the temperature had an effect on the smoothness of the peaks, on the solubility and on the time of analysis. Peak shapes could be improved by increasing the column temperature, since an increase of the temperature allowed to reduce the retention time (Fig. 3c). An operating temperature of 35 °C was found to be the optimal value for the detection of pleuromutilin, since it allowed to decrease and hence facilitate the flow of the mobile phase.



(a) (b) (c)

Fig. 3. Variation of the retention time according to the mobile phase (a), the flow rate (b) and the temperature (c)

Repeatability and linearity: Linearity of the analytical methods was tested by injecting standard solutions at four different concentrations. The relative standard deviation (RSD) of the peak area was 1.4 %. A good correlation was obtained between concentrations and peak area:

$$y = 6.642\ 10^4\ x + 6.81\ 10^5 \quad (1)$$

where y was the peak area and x was the concentration expressed in mg/mL; the regression coefficient R was 0.997.

Stability: The potential degradation of pleuromutilin during samples conservation in the mobile phase was carefully taken into account to determine the required conditions allowing to avoid this degradation. Samples were kept in the dark during

one month at room temperature and no debasement of pleuromutilin was noted (Table-3).

TABLE-3
STABILITY OF PLEUROMUTILIN AT ROOM TEMPERATURE

Time (days)	0	1	3	6	15	20	30
Pleuromutilin (g/L)	0.5	0.5	0.48	0.52	0.49	0.5	0.51

Modelling

Objective: Multiple linear regressions were the objective of the modelling to establish simple expressions involving dimensionless parameters to describe experimental data and to be readily considered by the users in the field.

Relation between the retention time, the flow rate and the mobile phase composition: The most suitable empirical equation proposed to estimate the retention time according to the flow rate and the mobile phase composition was given by the following equation:

$$t_R = P_1 * \exp(-P_2 * (A/B)) \quad (2)$$

The values of P_1 and P_2 for different flow rates are listed in Table-4 and Fig. 4 shows the variation of the computed values against the flow rate.

TABLE-4
COMPUTED VALUES OF THE PARAMETERS OF THE RETENTION TIME ACCORDING TO THE MOBILE PHASE COMPOSITION (PARAMETERS P_1 AND P_2 – eqn. 2) AND ACCORDING TO THE TEMPERATURE (PARAMETERS P_3 AND P_4 – eqn. 6)

Q (mL/min)	0.5	1	1.5	2
P_1	24.00	13.25	8.43	5.64
P_2	0.015	0.016	0.015	0.013
P_3	9.58	5.37	3.51	2.71
P_4	0.0006	0.0011	0.0018	0.002

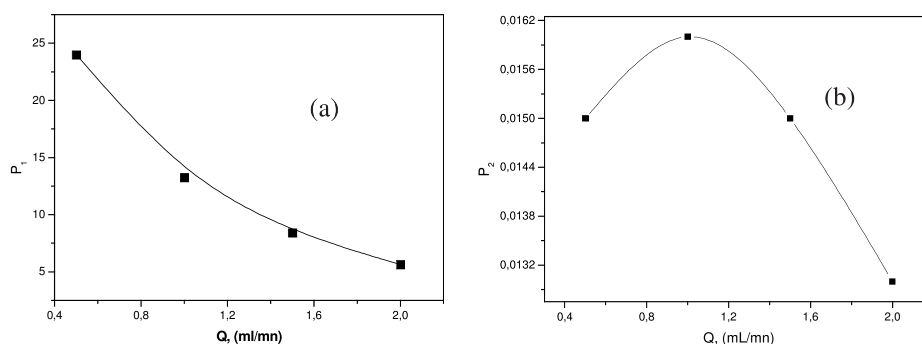


Fig. 4. Effect of the flow rate on parameters P_1 (a) and P_2 (b) of eqn. 2

The following correlations can be derived from the plot P_1 and P_2 *versus* the flow rate:

$$\text{For } P_1 \quad P_1 = a * \exp\left(\frac{b}{Q+c}\right) \qquad R^2 = 0.999 \tag{3}$$

$$\text{For } P_2 \quad P_2 = A + BQ + CQ^2 \qquad R^2 = 0.997 \tag{4}$$

The first set of retention experiences according to the flow rate and the mobile phase composition was then:

$$t_R\ (Q, A/B) = (a * \exp(\frac{b}{Q+c})) * \exp(-(A + BQ + CQ^2) * (A/B)) \tag{5}$$

The validation of this correlation is illustrated in Fig. 5a, shown by the high correlation coefficient ($R^2 = 0.996$).

For the validation of the above expression (eqn. 5), a three-dimensional simulation of equation 5 displayed in Fig. 5b may be helpful, showing that the retention time decreased with the flow rate and the mobile phase composition, with a more pronounced effect for the later parameter, due to an increase of the pressure at the inlet of the column. It was the consequence of an increase of the elution rate of the target compound, leading to a decrease of the retention time.

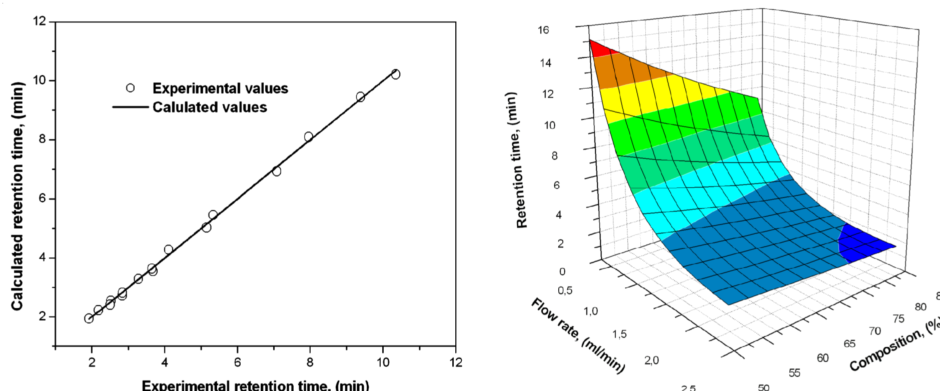


Fig. 5. Validation of the calculated retention time (eqn. 5) (a) and 3-D representation of the retention time according to the flow rate and the mobile phase composition (b)

Relation between the retention time, the flow rate and the temperature: After plotting the retention time according to the temperature for different flow rates, as shown in Fig. 3c for 2 mL/min for instance, the following empirical correlation can be deduced:

$$t_R = P_3 * \exp(-P_4 * T) \tag{6}$$

The values of P_3 and P_4 for different flow rates are collected in Table-4. Fig. 6 shows the variation of computed values against the flow rate. The following correlations can be derived since drawing P_3 and P_4 *versus* the flow rate:

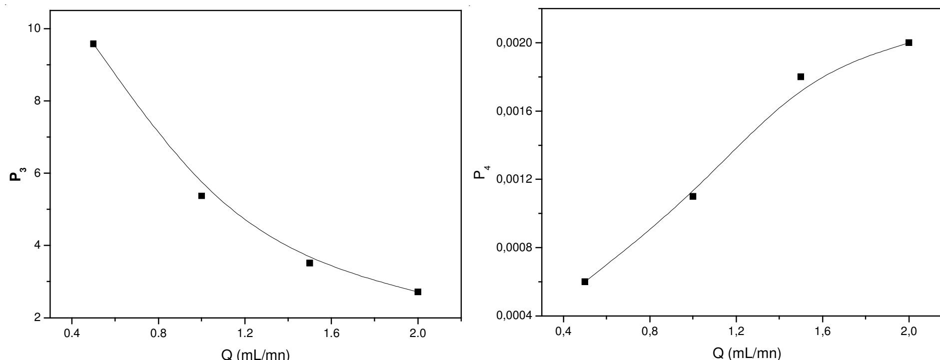


Fig. 6. Effect of the flow rate on parameters P_3 (a) and P_4 (b) of eqn. 6

$$\text{For } P_3 \quad P_3 = a + b\exp(-c * Q) \qquad R^2 = 0.993 \tag{7}$$

$$\text{For } P_4 \quad P_4 = A + BQ + CQ^2 \qquad R^2 = 0.979 \tag{8}$$

The first set of retention experience according to the flow and the temperature was then:

$$t_R\ (q, T) = (a + b\exp(-c * Q))\exp(-(A + BQ + CQ^2) * T) \tag{9}$$

The validation of this correlation was illustrated in Fig. 7a, also shown by the high correlation coefficient (R^2 = 0.999). A three-dimensional simulation of eqn. 9 shows that the retention time decreased with the temperature and the flow rate (Fig. 7b).

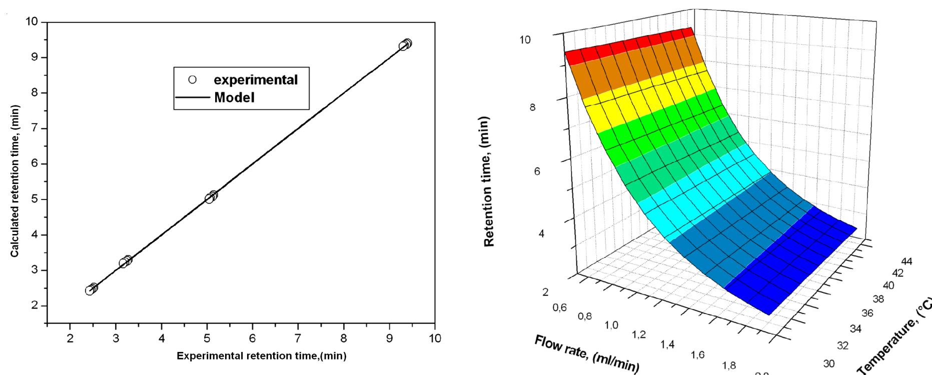


Fig. 7. Validation of the calculated retention time (eqn. 9) (a) and 3-D representation of the retention time according to the flow rate and the mobile phase temperature (b)

The temperature acts on the retention time by avoiding an overpressure at the inlet of the column, which increased the solubility of the solute in the mobile phase and decreased the viscosity of the mobile phase, leading to a reduction of the retention time.

Conclusion

Direct separation of pleuromutilin antibiotic extracted from biomass was achieved on C_8 column and acetonitrile/buffer in ratio of 60/40 as mobile phase, the retention time was 2.49 min at a temperature of 35 °C. The method was isocratic, simple and fast and optimized to be suitable for the separation of pleuromutilin residue.

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