# Determination of Phenicols Using a Fast and Reliable HPLC Method Developed on Hypercarb Stationary Phase

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*Hypercarb porous graphitic stationary phase was used to develop a fast and reliable method for simultaneous determination of chloramphenicol, thiamphenicol and florfenicol. The separation was achieved in 7 min elution being made isocratic using water modified with acetonitrile as mobile phase. The method was fully validated and showed good linearity, precision and accuracy. Limit of detection and quantification together with decision limit and detection capability were established. This method was applied for analysis of milk samples.*

*Keywords: chloramphenicol, thiamphenicol and florfenicol, Hypercarb, HPLC*

In veterinary medicine a great number of antimicrobial drugs are used against a variety of infectious agents in farm and companion animals. In the antimicrobial class of medicines amphenicols group has a distinct place. The earliest phenicol drug, a broad-spectrum antibiotic with outstanding antibacterial and pharmacokinetic properties is represented by chloramphenicol (CAP), isolated in 1947 from *Streptomyces venezuelae* and introduced into clinical practice in 1949 (as trade name Chloromycetin). Thiamphenicol (TAP) and florfenicol (FF) belong to the same group and are widely used in veterinary medicine due to their effective action against a broad variety of bacteria and anaerobic organisms. It was demonstrated that CAP causes blood dyscrasias, therefore it has been forbidden in some countries especially for the treatment of food-producing animals. However, it is still used in countries with low-income economies due to its advantages like relatively low cost, broad spectrum of action, and efficacy. Thiamphenicol and florfenicol are safer than CAP, but TAP is less effective, while florfenicol is more active and recommended even to treat bovine with respiratory diseases. Thiamphenicol is mainly excreted unchanged and is not easily metabolized in poultry, sheep, cattle or humans. Chloramphenicol is metabolized by the liver to chloramphenicol glucuronide (which is inactive) and a great quantity of the chloramphenicol is excreted as inactive metabolite by the kidneys. Because it was reported that chloramphenicol passes into breast milk, treatment during breast feeding should be avoided [1].

Despite the use of chloramphenicol is not allowed in human use, it is still widely used, even illegally in developing countries. In last years were reported findings of chloramphenicol residues in honey [2] and shrimps [3] imported from Asia.

Therefore, it is compulsory to have not only a regulatory control of both drugs and animal derived human food but also a veterinary legislation focused on biosecurity. Thus, many papers report analytical methods used for the quality control of phenicols, some of them are laborious and some involve quite expensive procedures and equipment. Among them, one can find immunoassays, microarrays, biosensors and microbiological-based methods for screening purposes [4], high-performance liquid chromatography, gas chromatography and their variants coupled with mass spectrometry [5-13]. Electrochemical and voltammetric methods have also been developed and applied for chloramphenicol determination [14,15]. In addition, while CAP and TAP have pharmaceutical monographs [16, 17], the quality control of florfenicol is not described in any official document.

Therefore, in this paper we present a new HPLC method developed on a Hypercarb porous graphitic carbon (PGC) suitable for determination of the three phenicols in various matrices.

## Experimental part

### *Chemicals*

Chloramfenicol (99.8%), thiamfenicol (99.6%) and florfenicol (99.5%) were purchased from Sigma-Aldrich. Acetonitrile and methanol were purchased from Sigma-Aldrich and were both of chromatographic purity.

### *Apparatus*

Water for chromatography (18 MΩ/cm) was obtained using an ULTRA CLEAR system (Richfield, USA).

Chromatographic analysis was performed using a HPLC system (Surveyor Plus, Thermo Electron Corporation, Waltham, MA) having a quaternary pump, an on-line degasser, an autosampler, temperature-controlled sample trays, a column thermostat, Photodiode Array (PDA) as detector and The ChromQuest as software.

A Jasco V-530 spectrophotometer (Tokyo, Japan) controlled by Spectramanager as software and provided with quartz cells of 10 mm were used for spectra recording.

Ultraviolet lamp 254/ 366 nm (Hanau Fluotest, HERAEUS - Germany) has been used for the forced degradation studies.

A centrifuge type Nuve NF 200 was used in this study.

### *Solutions*

Stock Solutions for HPLC

Stock solution of each phenicol was prepared in methanol for HPLC. A quantity of 10 mg of compound was exactly weighed and transferred each into a 10 mL

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volumetric flask filled up to the mark with methanol. The final concentration of each stock solution was: 1mg/mL for chloramphenicol, thiamphenicol and florfenicol, respectively.

Solution for system suitability: a volume of 1 mL of each stock solution of CAP, TAP and FF was quantitatively transferred into a 10 mL volumetric flask filled up to the mark with methanol for HPLC. In this solution, each phenicol is present in a concentration of 0.1 mg/mL.

*Working solutions for UV-Vis*

Working solutions using in validation studies were prepared from stock solutions for HPLC. Dilutions were made in methanol for HPLC.

*Forced degradation studies*

Forced degradation studies were carried out following the methodology as existing in literature [18] and the solutions tested were prepared as described for system suitability.

*Sampling*

Two types of drinking milk were sampled. First, we have investigated samples of commercially available products issued from the main companies existing on the Romanian market and second drinking milk from rural areas around Bucharest. Six breast milk samples were collected at hospital, according to recommended rules [19]. Adequate vials free from contamination were used. Milk samples were kept in the refrigerator (~ 4 ºC) and analysed within 72 h.

Samples for HPLC were prepared using a modified procedure existing in literature [20]. A quantity of 0.5 g of milk was added to a 1 mL of acetonitrile in a centrifuge tube. The mixture was centrifuged for 15 min at 5000 rpm. The supernatant was injected into HPLC. When necessary, a supplementary filtration through a 0.45 μm filter was made.

*Chromatographic conditions*

Chromatographic method was developed using a Hypercarb porous graphitic carbon (PGC) column (1004.6 mm and 5 μm particle size), purchased from ThermoElectron Corporation, USA. The separation was achieved isocratic, at 25ºC using a mobile phase of water and acetonitrile as organic modifier (40:60 v/v) pumped at a flow rate of 0.8 mL/min. The injection volume was 10 μL and the detection was made at 260 nm.

*Validation of the method*

International rules described in literature [21-23] were applied for the validation of the method. Parameters such as linearity, limit of detection and quantification, precision, accuracy and robustness were evaluated.

**Results and discussions**

We have decided to use a column packed with 100% porous graphitic carbon due to the amazing properties of this material. Commercially known as Hypercarb, this porous graphitic carbon (PGC) is well known for its particular structure which is designed by the hexagonal arrangement of carbon atoms that ensures a good separation of polar compounds having similar structure.

Therefore, we have thought that this stationary phase should be appropriate for the separation of phenicols, compounds with very similar in structure and properties (table 1). The development of this new chromatographic method took into consideration the literature existing for phenicols determination and also those existing on hypercarb column method development. Both aspects were important as there is not a well-established theory which could explain the retention of polar compound on a hypercarb stationary phase. The first choice for the organic modifier was acetonitrile and different mobile phase compositions were tested. The best results in term of resolution and symmetry were obtained using 60 % acetonitrile in water. The flow rate was 0.8 mL/min. Chromatogram obtained for this separation is presented in figure 1.

As one can observe the separation is achieved in 7 minutes with good resolutions among all compounds involved. The elution order was established separately injecting each solution of phenicol and it was: TAP (2.48 min), FF (3.21 min) and CAP (6.12 min). The resolutions between phenicols were 3.01 and 7.92 respectively. Even if there TAP and FF are quite similar in structure, a very good resolution was obtained. Being a separation on a PGC, a key factor that influences the elution is the geometry of the molecule. Therefore, we have computed the geometry of each phenicol using Scigress as software and observed

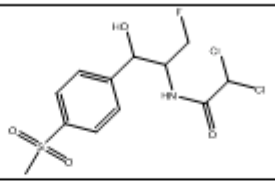
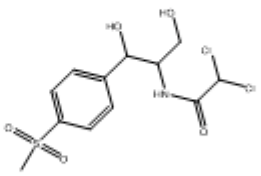
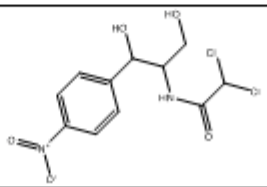
| Compound | Molecular formula | Chemical structure | $pK_a$ | logP |
|---|---|---|---|---|
| Thiamphenicol | $C_{12}H_{14}Cl_2FNO_4S$ |  | 9.76 | 1.14 |
| Florfenicol | $C_{12}H_{15}Cl_2NO_5S$ |  | 10.73 | -0.27 |
| Chloramphenicol | $C_{11}H_{12}Cl_2N_2O_5$ |  | 7.49 | -0.12 |

**Table 1**
CHARCTERISTICS OF THE PHENICOLS

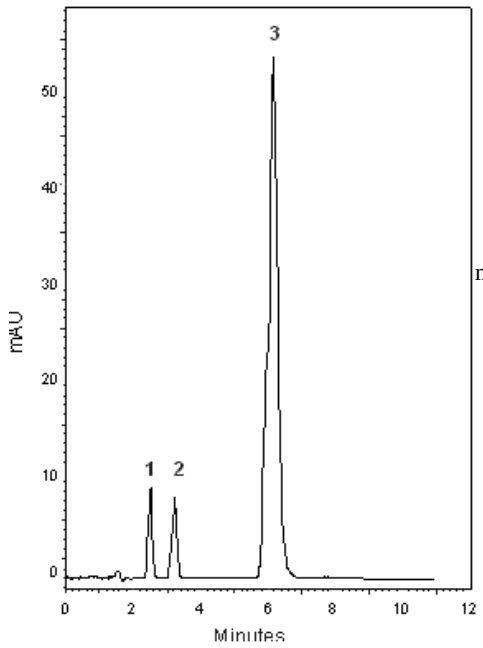



Fig. 1. A representative chromatogram of a mixture of phenicols (0.1 mg/mL): 1-thiamphenicol, 2-florfenicol, 3-chloramphenicol

that CAP, which has a nitrobenzene moiety has the greatest planar surface compared with two other compounds. In TAP and FF one can find the methyl sulfonyl group instead of nitrophenol group and this substituent contributes to a deviation from the planarity, which is greater in the case of TAP. Thus, the separation of phenicols is influenced by their geometries: the flatter the molecule, the greater the retention is.

Selectivity of this method was studied using solutions obtained in forced oxidative degradation conditions. Solutions of phenicols were kept at 254 nm and 366 nm for 8 h. Four new peaks were observed at 1.52, 1.96, 4.51 and 5.24 min when the solution was degraded at 254 nm, while at 366 only one impurity (1.96 min) was obtained. In both cases, the resolution between the impurity located at 1.96 min and TAP is 1.86.

*Method validation*

Linearity

Study of linearity was performed in a range of concentration established by regard to the regulated level of phenicols concentration. For instance, CAP has a 0.3 μg/kg [24] as minimum required performance limit (MRPL), while TAP has a maximum residue limit (MRL) in bovine milk set at 50 μg/kg. Regarding the FF, the literature suggests that there is any MRLP or MRL established. However, studies already published propose for FF a tolerated concentration in milk ranging from 0.048 to 1.22 mg/L [25].

Therefore, the linearity was checked for each phenicol taking into consideration the above mentioned concentrations. Results obtained are presented in table 2. Values obtained for intercept together with those for correlation coefficients indicate that this method should be considered validated for linearity.

*Limits of detection and quantification*

The experimental procedure involving the evaluation of signal to noise ratio was used to establish both limit of detection, LOD and limit of quantification, LOQ. Values obtained are presented in table 2.

*Decision Limit (CCα) and Detection Capability (CCβ)*

Both CCα and CCβ were calculated according to the Decision 2002/657/EC employing one of the procedures

| Compound | Linearity | | | LOD* μg/kg | LOQ* μg/kg | CCα μg/kg | CCβ μg/kg |
|---|---|---|---|---|---|---|---|
| | Slope | Intercept | Correlation coefficient | | | | |
| CAP | 93570 | 278.94 | 0.99981 | 0.008 | 0.032 | 0.3140 | 0.3310 |
| TAP | 7722.4 | -529.11 | 0.99967 | 0.020 | 0.080 | 53.23 | 57.22 |
| FF | 7538.1 | -1480.28 | 0.99965 | 0.040 | 0.160 | 51.98 | 53.77 |

**Table 2**
LINEARITY AND SENSITIVITY OF THE HPLC METHOD

**Determined in milk matrix*

| Compound | Quantity μg/kg | Recovery [% ± s.d.] | Precision (R.S.D., %) intra-day | Precision (R.S.D., %) intra-day |
|---|---|---|---|---|
| Thiamphenicol | 25 | 97.96 ± 1.80 | | |
| | 50 | 100.6 ± 2.03 | 4.04 | 2.77 |
| | 75 | 98.29 ± 4.66 | | |
| Florfenicol | 25 | 94.02 ± 3.71 | | |
| | 50 | 98.52 ± 1.78 | 2.63 | 3.47 |
| | 75 | 95.17 ± 5.24 | | |
| Chloramphenicol | 0.15 | 94.23 ± 0.0104 | | |
| | 0.30 | 96.62 ± 0.0059 | 2.05 | 3.14 |
| | 0.45 | 96.74 ± 0.0152 | | |

**Table 3**
PRECISION AND ACCURACY OF THE HPLC METHOD

described in literature [26]. For all analytes 20 blank drinking milk samples were spiked with phenicols as it follows: chloramphenicol at 0.3 μg/kg while thiam-phenicol and florfenicol at 50 μg/kg. The results obtained are presented in table 2.

*Recovery*

Fortified milk samples were used to establish the recovery of these phenicols. Three levels at 0.5, 1 and 1.5 times the permitted limit of concentration were considered in this study. Recoveries were calculated and presented in table 3. As one can observe the method has recoveries ranging from 94.02 to 100.6% which are in agreement with data existing in literature [13].

*Precision*

The relative standard deviation (R.S.D. %) was calculated for milk samples fortified with phenicols, and repeatability and intermediate precision were estimated. First, six identical solutions containing TAP and FF each 50 μg/kg, and CAP 0.3 μg/kg were analysed. Second, in the following day a different analyst applied the HPLC method on samples as described for repeatability. Results presented in table 3 confirm a good precision for this chromatographic method.

*Stability of solutions*

Different stock solutions of phenicols were kept in dark or light, at room temperature or at 4°C and analysed one, two, three and four weeks after preparation. The assay performed on the solutions kept in refrigerator showed that they are the most stable, with a maximum loss of 1.87% (for FF), and a minimum for CAP (0.54%).

*Application of the method on milk sample*

Various drinking milk samples from both cattle and sheep were included in our study. We have analysed samples for from five Romanian companies, two from European companies, four collected from local market and originated from Bucharest's rural area. In addition, in this study we have included six breast milk samples, collected from mothers living in the rural area around Bucharest, as their lifestyle are quite different from people living in the city and their eating habits are based on self-produced food. Each sample was analysed in triplicate and the data obtained showed that all of them are free of phenicols. However, a composite sampling design [27] was applied on both drinking milk and breast milk to obtain only two representative samples. These two samples were spiked with known concentrations of phenicols (at MRPL value for chloramphenicol, and MRL value for both florfenicol and thiamphenicol) and analysed in triplicate using this HPLC method. Recoveries (%) calculated were 94.23±5.03 for thiamphenicol, 95.07± 2.75 for florfenicol and 98.41± 3.78 for chloramphenicol.

## Conclusions

A new HPLC method has been developed on a porous graphitic carbon (PGC) stationary phase able to separate chloramphenicol, thiamphenicol and florfenicol in less than 7 minutes. This method was fully validated and applied on milk samples. The data obtained in the validation study suggest that this method could be used for florfenicol assay in veterinary drug (for in-process and finished product control) as this compound does not exist in any pharmacopoeia monograph.

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