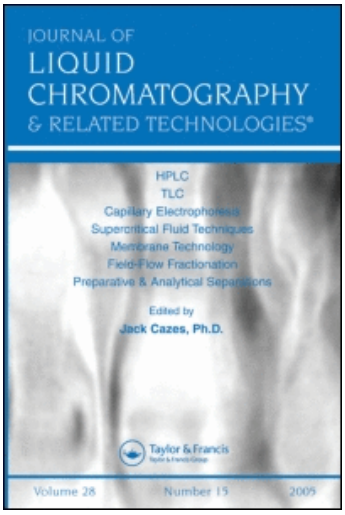

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ASSAY OF CHLORAMPHENICOL IN BIOLOGICAL MEDIA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH U.V. ABSORBANCE AS THE DETECTION MODE

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ABSTRACT

Measurement of chloramphenicol in plasma, milk, urines and tissues was realized by high-performance liquid chromatography, with U.V. absorbance as the detection mode, at a wavalength set at 280 nm.

Our method met requirements of sensitivity set up by the Ministries : 0.02 p.p.m. for meat and offal, and 0.01 p.p.m. for milk.

The regression analysis, realized for each standard curve by expressing the chromatographic peak heights as a function of chloramphenicol levels in plasma, milk or urines, or chloramphenicol quantities in tissues were linear as far as our concentration range is concerned.

The variation coefficients, determined by the repeatability assay on 10 samples at different concentrations were 1.37 to 13.29 ug/ml.

INTRODUCTION

Since its introduction in 1949 chloramphenicol has been extensively used because of its effective antibiotic activity against a wide range of bacteria. It is useful in human therapeutic for the treatment of Gram-negative bacterial infections especially typhus and meningitis caused by Hemophilus influenzae type b resistant to ampicillin. It is commonly used in many countries for the treatment of infection in domestic animals produced for food consumption because of the efficacity of the antibiotic and the relatively inexpensive availability (1).

However chloramphenicol is responsible for two types of adverse effects : marrow aplasia, unrelated to dosage, and dyserythropoiesis dose related ; serum chloramphenicol concentrations of greater than 25 ug/ml and prolonged courses of therapy have been associated with bone marrow depression and fatal toxicities, such as "gray syndrome" in neonates and "gray toddlers syndrome" in infants have been reported with serum concentrations above 50 ug/ml (2).

To protect the consumer, zero tolerance levels have been proposed for chloramphenicol in edible tissues and milk (3). The chloramphenicol concentration in the plasma of the patient under treatment is also to be measured.

A sensitive and specific method to detect chloramphenicol is of primary importance in helping to monitor and reduce the potential incidence of this residue in the food chain.

The most common method used to detect chloramphenicol is the bioassay (4) but this method offers limited sensitivity and lack of specificity when samples contain other microbial inhibitors. Colorimetric methods and adaptations measure compounds formed after reduction and diazotation of the choramphenicol, so they are not specific (5). Gas-liquid chromatography with electron capture spectrometry methods of analysis (6, 7, 8) and competitive enzyme-linked Immunoassay (1) have greatly increased the specificity and sensitivity of chloramphenicol assays in biological samples.

These methods are not easy to perform and use ex-pensive materials. Several techniques using high performance liquid chromatography (HPLC) have been employed to quantitate chloramphenicol in biological fluids (2 - 9 - 10 - 11 - 12 - 13 - 14 - 15 - 16 - 17). Some methods permit the simultaneous measurement of chloramphenicol and its succinate es-ters in serum, plasma or urine and others offer a good sen-sitivity, but none allows both sensitivity and specificity in plasma, urine, tissue and milk. We developed a high per-formance liquid chromatographic technique with U.V.

absorbance as the detection mode at a wavelength set at 280 nm.

MATERIAL AND METHODS

The assay was realized with a high-performance liquid chromatographic technique using U.V. absorbance as the detection mode.

Material

The chromatograph used was equipped with the following devices :

- a F 6000 A pump, WATERS ASS. France,
- a uBondapak C_{18} column, 10 um particle size, WATERS ASS.,
- an automatic sample injector WISP 710 A, WATERS ASS.,
- a fixed-wavelength ultraviolet detector, M 440, set at 280 nm, WATERS ASS.
- an Omniscribe recorder, HOUSTON INSTRUMENT.

Chromatographic Conditions

The technique used was based on reversed-phase partition chromatography:

- Mobile phase:
  - * acetonitrile.....300 ml
  - * aceto-acetic buffer 0.01 N s.q.for.....1000 ml

- Aceto-acetic buffer 0.01 N :

  * solution A :

  sodium acetate 0.01 N(0.82 g/l).....166 ml

  * solution B :

  acetic acid 0.01 N (0.6 g/l).....1000 ml

- Flow rate : 1 ml/min
- Chart speed : 2.5 mm/min
- Injection volume : 5 to 30 ul.

Drug Analysis

a) assay in plasma

One ml of plasma was introduced into a 10 ml glass tube (SVL screw-tubes with teflon caps) and supplemented with 6ml ethyl acetate,(Ethyl Acetate MW = 88.10 g/mol; d = 0.90; quality for analysis MERCK). This mixture was shaken for 5 min and then centrifuged for 10 min at 4000 r.p.m. Five ml of the upper organic layer were transferred into a clean screw-capped tube and evapored to dryness under a stream of nitrogen. The dry residue was then dissolved in 200 ul methanol (Methanol, M = 32.04 g/mol; d (from 4 to 20°C) = 0.791 to 0.792; quality for analysis, MERCK),10 to 20 ul of which were injected into the chromatograph.

b) assay in milk

Chloramphenicol was extracted from milk with 10 ml ethyl acetate per aliquot of 2 ml.

c) assay in the urines

Each urine sample was assayed in duplicate ; the first assay was perfomed before,the other after hydrolysis with Helix Pomatia (Helix Pomatia, 100 000 units of ß-glucuronidase, 1.000.000 units of sulfatase, reagent IBS). We were able to measure free chloramphenicol in 1/15 dilution of the urines ; 15 to 30 ul were injected directly into the chromatograph. The purpose of hydrolysis with Helix Pomatia was to liberate sulfo- or glycuronoconjugated chloramphenicol thus enabling measurement of total chloramphenicol. The procedure involved pipetting 1 ml of urine in a 10 ml screw-capped tube, to wich were added 3 drops of Helix Pomatia; the mixture was shaken and then kept standing for 12 hours at 37°C. 400 ul of this preparation were transferred into a clean screw-capped tube, supplemented with 1.6 ml of chloroform/isopropanol (50/50; V/V) (Chloroform, MW = 119.38 g/mol; d = 1.49; quality for HPLC, CARLO ERBA; isopropanol, M = 60.10 g/mol; d = 0.78; quality for analysis MERCK). After vortexing (Vortex mixer BIOBLOCK) for 1 min, and centrifuging 10 min at 3500 r.p.m.,1 ml of the lower organic layer was evapored to dryness under nitrogen, and the residue dissolved in 200 ul of methanol, 15 to 30 ul of which were injected into the chromatograph.

d) assays in the tissues

The tissue samples were weighed (weight varying from 250 mg to 1 g) and introduced into 25-ml screw-capped tubes containing 5 ml of sodium chloride solution at 0.9 % (LAVOISIER), before being grinded with an Ultra Turrax (homogenizer 20 000 r.p.m., 75 W Ultra Turrax) for 5 min.

Six ml of ethyl acetate were then added to the tissue homogenates. The mixture was shaken for 5 min, and then centrifuged for 5 min at 3500 r.p.m.. Five ml of the upper organic layer were washed and introduced in a clean screw-capped tube of 10 ml.

The procedure involved a second extraction of the tissue homogenates, identical to the first one ; 5 ml of the organic layer were added to the first series, followed by evaporation to dryness under a stream of nitrogen. The residue was dissolved in 1 ml of methanol, and then supplemented with 2 ml of distilled water. The entire content was introduced into a 1-cm diameter column, filled up to 2 cm with resin (Carboxymethyl Sephadex) suspended in distilled water : the impurities were fixed by the resin, that was successively eluted by 3 ml, 2 ml and 1 ml of distilled water.

Two other extractions were realized on the eluate with 6 ml of ethyl acetate each time. The organic phase (twice 5 ml) was transferred in a 10

ml screw-capped tube and evapored to dryness under a stream of nitrogen. The residue was dissolved in 150 ul of methanol and injected into the chromatograph.

Calibration

Standard samples were realized from methanolic solutions of chloramphenicol titrating 500 to 50 ug/ml. The daughter solutions were obtained by geometrical dilution (basis 2). 50 ul of these solutions were added, either to 950 ul of plasma coming from a standard pool of plasma, or to 950 ul of milk, 950 ul of urine or to a tissue sample weighing from 500 mg to 1 g and immersed in 5 ml of a sodium chloride solution at 0.9 %.

The standards thus obtained were used to construct the calibration curve and processed in the same way as the other samples.

RESULTS

Examples of chromatographic tracings are shown in figure 1.

For each standard curve, we realized a regression analysis by expressing the chromatographic peak heights as a function of chloramphenicol levels in plasma, milk or urines, or chloramphenicol quantities in tissues.

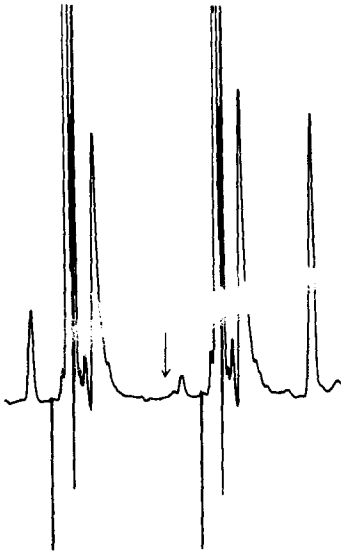

FIGURE 1 : Chromatograms corresponding to blank urine and urine sample (post hydrolysis) of chloramphenicol (injection volume : 30 ul ; sensitivity : 0.05 AUFS ; flow rate : 1.4 ml/min).

Curves were realized for plasma with 3 concentration levels (25 to 1 ug/ml, 7.5 to 0.039 ug/ml and 1000 to 15.6 ng/ml), for milk with 2 concentration levels (10 to 0.078 ug/ml and 160 to 10 ng/ml), for the other samples with one concentration level (non hydrolysed urines : 50 to 0.8 ug/ml ; hydrolysed urines : 100 to 1.6 ug/ml ; heart tissue : 1280 to 20 ng/ml).

The regression curves are linear as far as our concentration range is concerned and the straight line equations are the following :

- plasma calibration :

Fig. 2 : h = 10.6118 C_{plasm} - 0.4358 r = 0.9997
(mm) (ug/ml) (5 degrees of freedom)

Fig. 3 : h = 29.8114 C_{plasm} + 0.3478 r = 0.9991
(mm) (ug/ml) (8 degrees of freedom)

Fig. 4 : h = 0.1067 C_{plasm} - 0.1582 r = 0.9998
(mm) (ug/ml) (6 degrees of freedom)

- milk calibration :

Fig. 5 : h = 13.6023 C_{milk} - 0.9995 r = 0.9995
(mm) (ug/ml) (8 degrees of freedom)

Fig. 6 : h = 0.3640 C_{milk} - 0.0571 r = 0.9991
(mm) (ng/ml) (4 degrees of freedom)

- urine calibration :

* prior hydrolysis

Fig. 7 : h = 2.0503 C_{urine} + 0.4321 r = 0.9987
(mm) (ug/ml) (6 degrees of freedom)

* post hydrolysis

Fig. 8 : h = 1.6640 C_{urine} - 0.0328 r = 1.0000
(mm) (ug/ml) (6 degrees of freedom)

- heart tissue calibration :

Fig. 9 : h = 0.2003 Q_{tissue} - 2.3548 r = 0.9969
(mm) (ng) (6 degrees of freedom)

The variation, within one day, was estimated from the variation coefficients determined by the repeatability assay on 10 samples at different concentrations; these coefficients are described in table I :

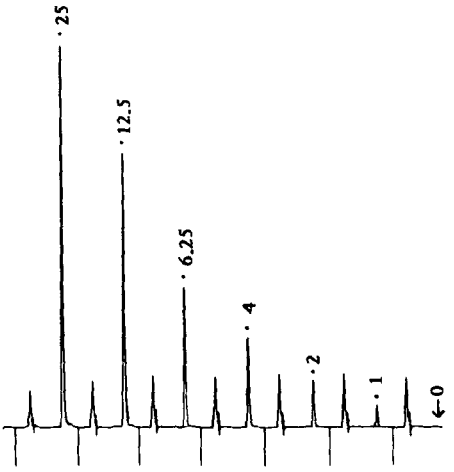

FIGURE 2 : Chromatograms corresponding to plasma calibration of chloramphenicol - ug/ml (injection volume for the first sample : 10 ul and for the others : 15 ul ; sensitivity : 0.1 AUFS ; reducing scale : 0.44).

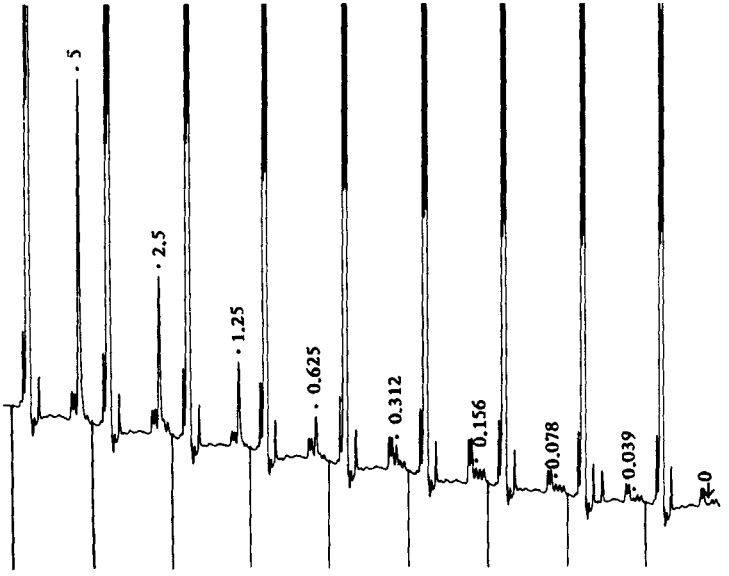

FIGURE 3 : Chromatograms corresponding to plasma calibration of chloramphenicol - ug/ml (injection volume : 10 ul ; sensitivity : 0.01 AUFS ; reducing scale : 0.44).

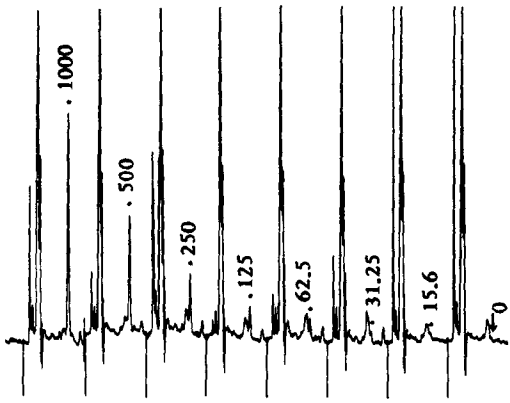

FIGURE 4 : Chromatograms corresponding to plasma calibration of chloramphenicol - ng/ml (injection volume : 15 ul ; sensitivity : 0.01 AUFS ; reducing scale : 0.44).

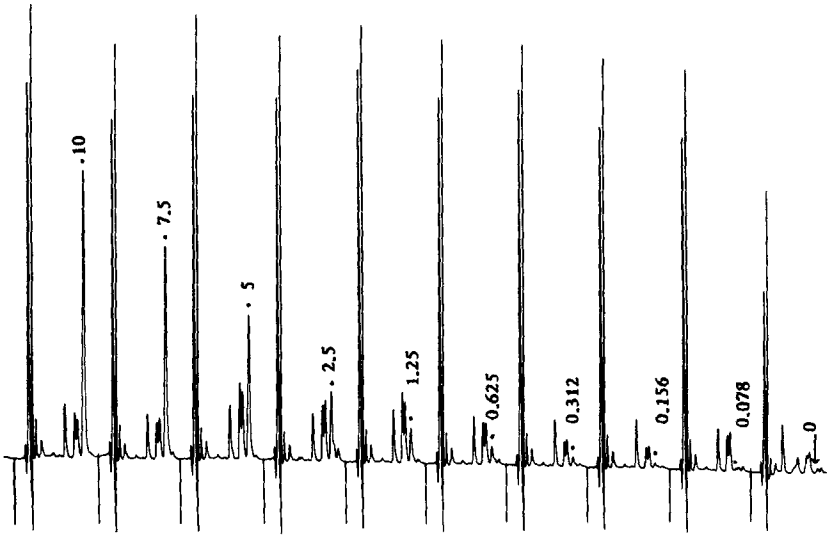

FIGURE 5 : Chromatograms corresponding to milk calibration of chloramphenicol - ug/ml (injection volume : 10 ul ; sensitivity : 0.02 AUFS ; reducing scale : 0.44).

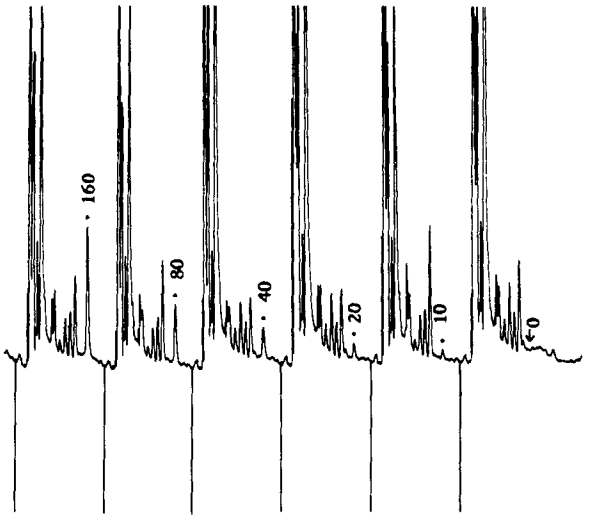

FIGURE 6 : Chromatograms corresponding to milk calibration of chloramphenicol - ng/ml (injection volume : 15 ul ; sensitivity : 0.01 AUFS ; reducing scale : 0.44).

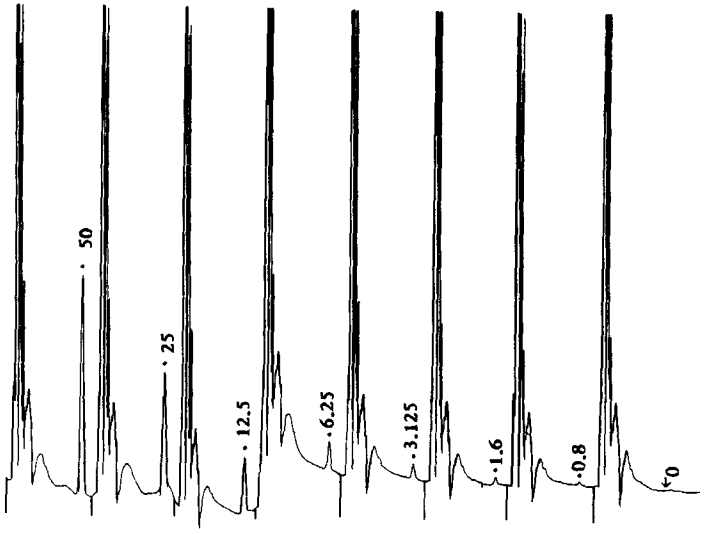

FIGURE 7 : Chromatograms corresponding to urine calibration of chloramphenicol prior hydrolysis - ug/ml (injection volume : 15 ul ; sensitivity : 0.05 AUFS ; reducing scale : 0.44).

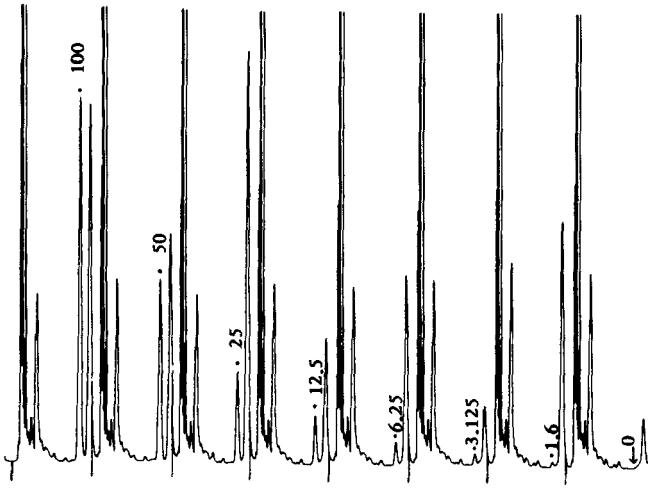

FIGURE 8 : Chromatograms corresponding to urine calibration of chloramphenicol post hydrolysis - ug/ml (injection volume: 30 ul ; sensitivity : 0.2 AUFS ; reducing scale : 0.38).

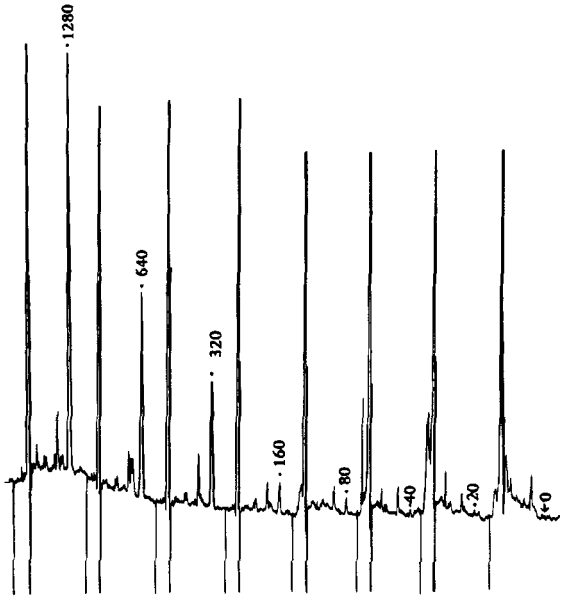

FIGURE 9 : Chromatograms corresponding to heart tissu calibration of chloramphenicol - ng (injection volume : 10 ul ; sensitivity : 0.005 AUFS ; reducing scale : 0.38).

TABLE 1

VARIATION COEFFICIENTS DETERMINED BY THE REPEATABILITY ASSAY

BIOLOGICAL MEDIA	CONCENTRATIONS OR QUANTITIES	VARIATION COEFFICIENTS (%)
PLASMA	5 ug/ml	1.41
	1 ug/ml	6.19
	0.312 ug/ml	6.69
MILK	1 ug/ml	13.29
	0.1 ug/ml	12.12
NON HYDROLYSED URINES	25 ug/ml	1.37
	3.125 ug/ml	3.4
MUSCULAR TISSUE	1 ug/ml	7.39
	0.3125 ug/ml	7.01

Examples of chromatographic tracings corresponding to repeatability at a concentration of 0.312 ug chloramphenicol per ml of plasma and 0.1 ug per ml of milk are illustrated in figures 10 and 11.

The sensitivity thresholds, observed in plasma and milk are 20 and 10 ng/ml respectively. In the tissues, the threshold is equal to 20 ng chloramphenicol per trial sample. In the urines, the thresholds in the analytic conditions are 0.5 ug chloramphenicol per ml prior hydrolysis and 1 ug per ml post hydrolysis.

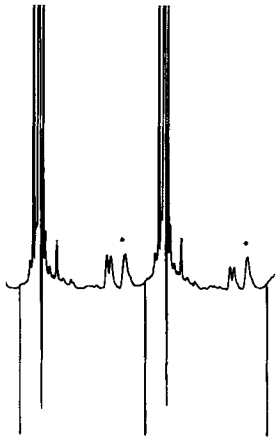

FIGURE 10 : Chromatograms corresponding to repeatability assay for concentration values equal to 0.31 ug of chloramphenicol per ml of plasma (injection volume : 10 ul ; sensitivity : 0.01 AUFS).

DISCUSSION AND CONCLUSION

Many methods of chloramphenicol assay have been described and the characteristics of some of them are indicated in the table II.

Our method offers a sensitivity threshold very low; the requirements of Ministry are met so it is possible to use our method for the detection of chloramphenicol in milk and tissue in food producing animals. This method is also adaptable to the routine analysis of a large number of samples such as plasma or urine or milk for the pharmacokinetic studies or clinical use.

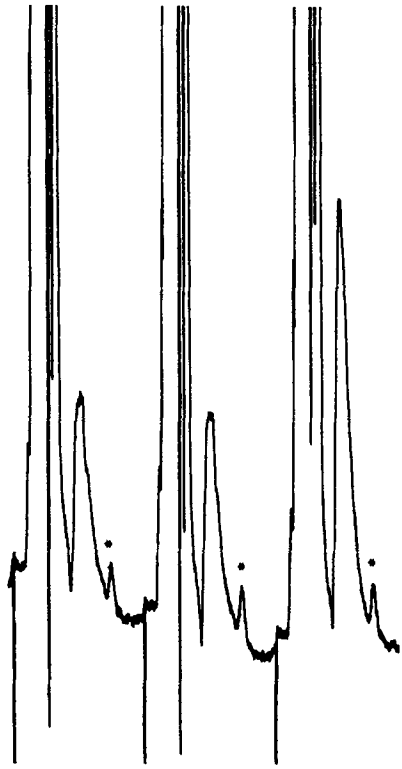

FIGURE 11 : Chromatograms corresponding to repeatability assay for concentration values equal to 0.1 ug of chloramphenicol per ml of milk (injection volume : 40 ul ; sensitivity : 0.01 AUFS).

This technique is accurate and precise as shown by the correlation coefficients of the regression analysis calculated for the standard curves and by the data of the repeatability tests. The low intercept values of the regression standard curves allow to say that the assay technique is specific and exclude a significant interference by endogenous compounds and

TABLE 2

CHARACTERISTICS OF CHLORAMPHENICOL ASSAYS

REFERENCE	DRUG	BODY FLUID OR TISSUE	ASSAY METHOD	LIMIT OF DETECTION
4	CAP	Milk	Bioassay	2 to 10 ug/ml
5	CAP	Milk	Colorimetric detection after preparation of cation exchanger-albumina column	> 5 ppb
1	CAP	Muscle tissue	Enzyme-linked Immunoassay CELIA	1 ng/ml
6	CAP	Liver muscle	GCMS	5 ppb
7	CAP	Muscle liver kidney	GCMS (electron capture detection)	5 ng/g
8	CAP	Milk	GCMS (electron capture detection)	10 ppb
9	CAP - CAPS	Serum urine	HPLC	not described
10	CAP-CAPS-CAPG	Urine	HPLC	not described
11	CAP	Serum	HPLC	not described
12	CAP-CAPS	Serum	HPLC	0.5 and 0.2 ug/ml
13	CAP-CAPS-CAPP	Serum	HPLC	a few ng/ml
2	CAP-CAP-1-S-CAP-3-S	Serum	HPLC	0.5 - 1.5 - 1.2 ug/ml
14	CAP-CAPS	Plasma	HPLC	0.5 - 1 ug/ml
15	CAP	Serum	HPLC	0.25 ug/ml
16	CAP	Milk	HPLC	5 ppb
17	CAP	Serum	HPLC	not described

CAP = Chloramphenicol
CAPS = Chloramphenicol succinate
CAPG = Chloramphenicol glucuronide
CAPP = Chloramphenicol palmitate
CAP-1-S = Chloramphenicol-1-monosuccinate
CAP-3-S = Chloramphenicol-3-monosuccinate

the chromatography permits to separate chloramphenicol from any other drugs.

Moreover, this method offers the possibility of conjugated chloramphenicol in urine.

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