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DETERMINATION OF IVERMECTIN IN MILK BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Résumé

DÉTERMINATION DE L'IVERMECTINE DANS LE LAIT PAR CHROMATOGRAPHIE LIQUIDE HAUTE PERFORMANCE. — Une technique de dosage de l'ivermectine dans le lait a été développée. Les échantillons de 1 ml de lait sont soumis successivement à une étape d'extraction par solvants organiques (iso-octane et acétonitrile) puis à une chromatographie de phase inversée dans un système de chromatographie liquide haute performance (CLHP). Cette procédure a été validée par des études de récupération et de précision. Dans le domaine de la spécificité, l'analyse par spectrométrie de masse de fractions collectées à la sortie du chromatographe a permis de confirmer l'identité du composé dosé. Une étude pharmacocinétique réalisée sur une vache ayant reçu une dose de 0,2 mg/kg d'ivermectine a montré le passage et la persistance de cette substance dans le lait pendant une période d'environ trois semaines, le niveau de concentration étant voisin de celui obtenu dans le plasma. Le seuil de détection de la méthode a été établi à 2 ng/ml.

Ivermectin, a potent antiparasitic agent derived from naturally occurring fermentation products (Miwa *et al* 1983), exhibits a broad spectrum of activity at a very low dosage level eg, ivermectin B1a (fig 1) is effective against a wide range of helminths in sheep and cattle at a single oral or parenteral dose of 0.1 mg/kg (Ergeton *et al* 1979).

Bioavailability and pharmacokinetics studies based upon plasma analyses have been performed (Prichard *et al* 1985, Lo *et al* 1985) and results indicated that ivermectin was very slowly eliminated with a terminal half-life in the range of 5-7 days. Tissue distribution of ivermectin residues in cattle and sheep have been studied (Tway 1981) and it was shown that the major simple component in the edible tissue of the animals was the unaltered drug. In this respect, the liver is the tissue in which the drug is most persistent, residues being detected over a period of 28 days post-administration.

The passage of ivermectin from blood to milk has not been demonstrated and the purpose of this study was to develop a rapid, simple and sensitive method for the determination of ivermectin in milk.

This paper describes a relatively simple and accurate method for the determination of ivermectin in milk by extraction clean-up and direct injection onto a reversed phase system with UV detection.

Materials and Methods

Chemicals and reagents

All reagents were analytical grade purity; methanol and acetic acid were purchased from Merck (Darmstadt, FRG). Acetonitrile originated from Farmitalia-Carlo erba (Milan, Italy). Water was desionized and distilled. Ivermectin standard was kindly provided by Dr B Robin (Merck Sharp and Dohme, Paris)

Apparatus

A constant flow high performance liquid chromatograph (Waters Assoc, Milford, MA, USA) consisting of a model M45 pump and a model M481 spectrophotometer. The column (150 × 3.9 mm ID) was packed with a 5 µm reversed phase silica, chemically bonded with octadecyl silane (Resolve, Waters Assoc, Milford, MA, USA).

Standards

A stock solution of ivermectin 1.0 mg/ml was prepared in methanol. For recovery experiments, various standard solutions of ivermectin in methanol were prepared. The stock solution and all standard solutions were stored at about 4 °C.

Operating conditions

The mobile phase was 2 % glacial acetic acid in water, methanol, acetonitrile (90 ml/10 ml/250 ml,v/v/v). Before use, the mobile phase was degassed by applying vacuum to the solvent reservoir for approximately 5 min.

Table 1. — Recovery of ivermectin from milk

Ivermectin Amount added to milk ng/ml	Number of determination	Recovery (a) (% ; mean \pm SD)	Coefficient of variation (%)
20	5	73.6 \pm 2.1	2.8
40	5	79.4 \pm 2.4	3.0
60	5	81.2 \pm 2.6	3.2
80	5	79.3 \pm 4.1	5.1
100	5	80.8 \pm 5.3	6.5
Mean		78.9 \pm 3.6	4.1

(a) The percentage recovery was determined by comparing the peak height of ivermectin extracted from samples with peak height obtained by direct injection of standard solutions.

The system was operated at ambient temperature at a flow rate of 1.5 ml/min; the wavelength of the UV detector was set at 246 nm.

Extraction procedure

In a corex tube of 30 ml, 1 ml of milk, 5 ml of acetone-water (acetone: 2.5 ml, water: 2.5 ml) were mixed, placed in an ultrasonic bath for 5 min and then extracted by two 5 ml portions of iso-octane by shaking and centrifuging. The iso-octane extracts were combined and evaporated to dryness in a 80 °C water bath under a stream of dry nitrogen. The residue was redissolved in

8 ml of acetonitrile, mixed and centrifuged 5 min at 5 000 g; the lipidic residue was discarded, the organic layer was transferred to a clean 15 ml glass tube and then extracted by two 3 ml portions of hexane. The acetonitrile phase was decanted into another tube and evaporated to dryness using a gentle stream of nitrogen.

Calibration curve

The calibration curve was constructed by plotting the peak height versus the concentrations. The concentration of unknowns were calculated from peak height ratio by interpolation of the calibration curve.

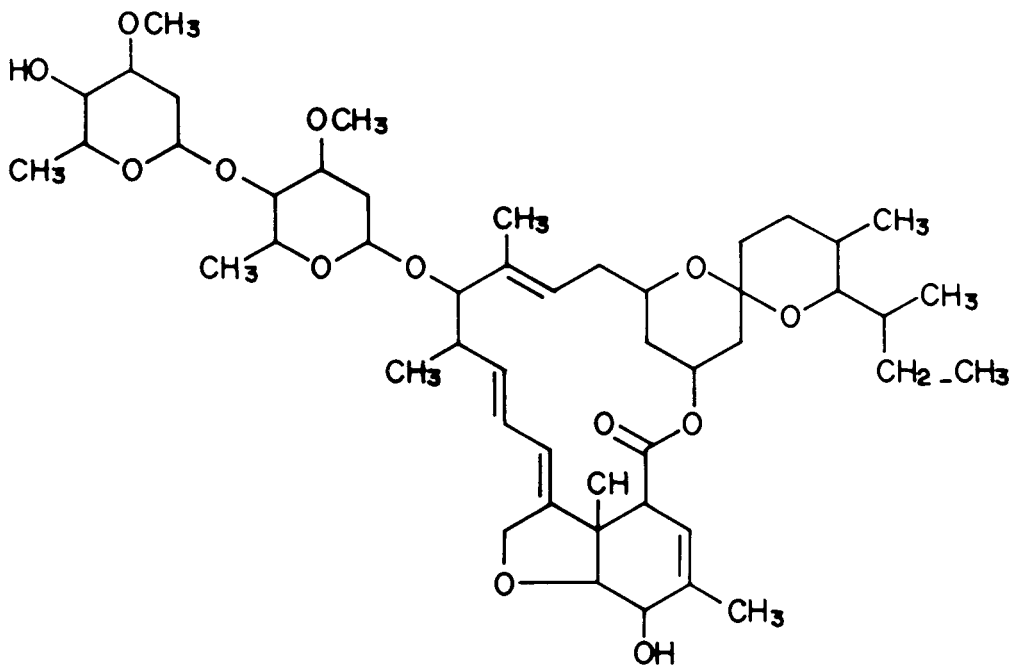


Fig 1. — Chemical structure of ivermectin B1a

Recovery study

To measure recovery, various concentrations of ivermectin were added to milk and the samples were extracted as described above. The percentage recovery was determined by comparing the peak height of ivermectin extracted from samples with peak height obtained by direct injection of standard solutions.

Kinetic study

The method has been applied to quantitate ivermectin residues in the milk of a cow. The animal was injected subcutaneously with a solution of ivermectin at a dosage level of 0.2 mg/kg. Samples of blood and milk were collected over the period of 3 weeks and were stored at -20°C until analysis.

Results

Evaluation of the method

Analyses of the standards with or without extraction showed in both instances a high corre-

lation between the concentration (x) and peak height (y). The equation of a typical calibration graph from 10 ng to 100 ng/ml was $y = 0.121x - 0.15$ and the correlation coefficient was 0.999.

The average recovery of milk samples spiked with 20-100 ng/ml corresponded to $78.8 \pm 3.3\%$ (mean \pm SD; n = 5) (table 1).

The precision of the extraction procedure and chromatography was evaluated by processing as replicates on different days, aliquots of pooled milk samples containing 60 ng/ml; the inter assay coefficient of variation was 3.2%.

Representative chromatograms milk samples are shown in figure 2. In the chromatogram obtained from a blank milk sample (fig 2B) no peak are present which might interfere with the determination of ivermectin. Figure 2C shows a chromatogram of an extract of milk spiked with 40 ng/ml ivermectin. Analytical reference standard of

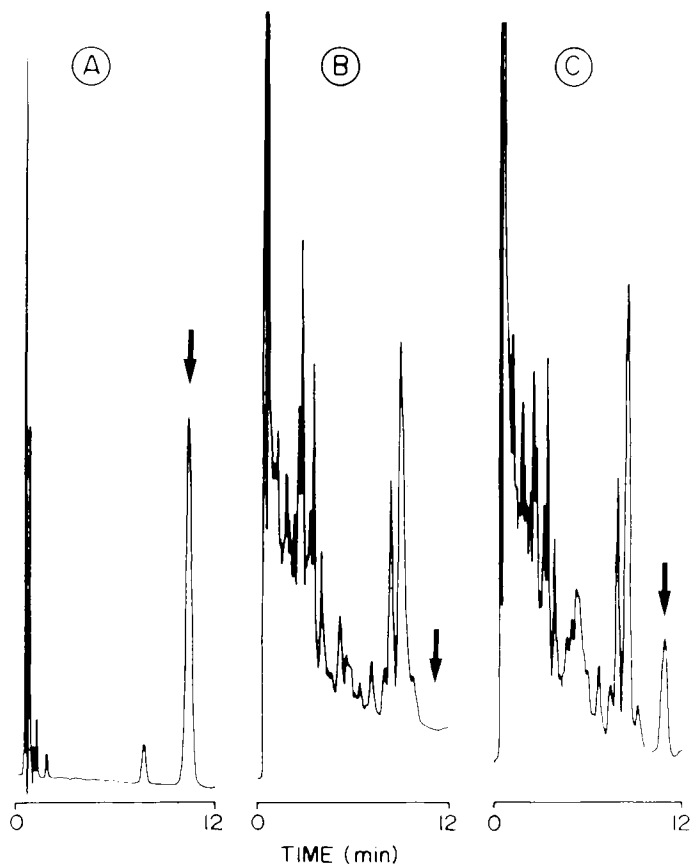


Fig 2. - (A) chromatogram of a standard, 100 ng ivermectin B1a; (B) chromatogram obtained from a blank milk sample; (C) chromatogram of an extract of milk spiked with 40 ng/ml ivermectin.

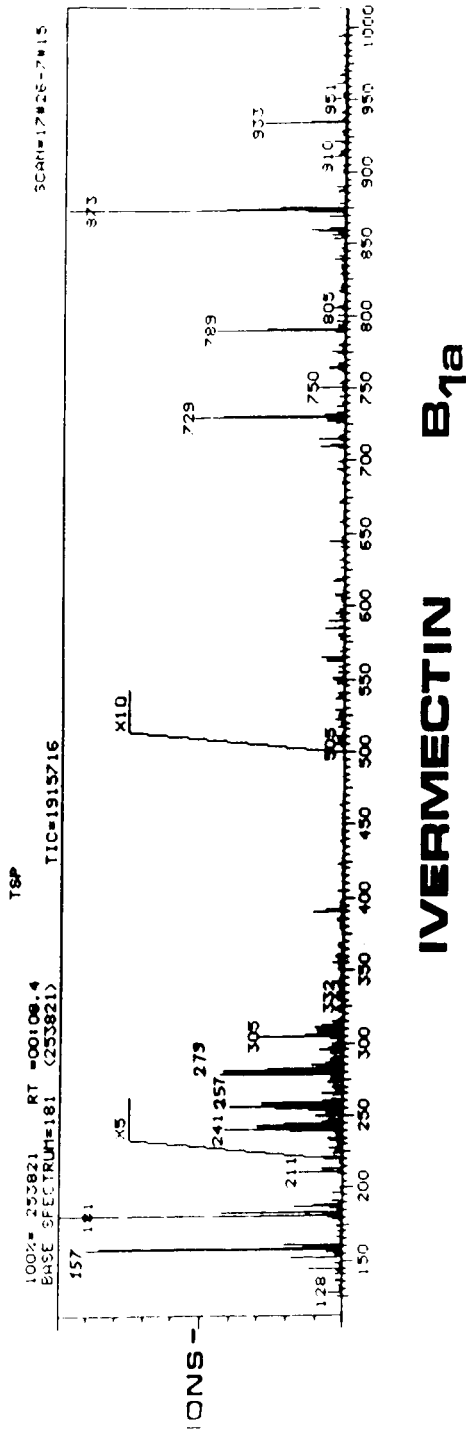


Fig 3. - Mass spectrum of Ivermectin B1a using negative ions detection.

100 ng of ivermectin is shown in figure 2A. The retention time of ivermectin was 11 min.

Responses were considered significant when the signal to noise ratio was > 2 ; in such conditions, using a detector sensitivity of 0.005, levels as low as 2 ng/ml giving peak height of 0.5 cm could be quantitated.

The specificity of the analysis and the identity of the ivermectin peak was confirmed using mass spectrometry (M. Lesieur, 1986, personal communication). Mass spectrometry pattern of ivermectin B1a was determined by direct injection (thermospray filament-off) of standard using negative ions detection. Ivermectin gave base peak at m/e 983 and 873 corresponding to the molecular ions $m\text{-CH}_3\text{COO}^-$ and $m\text{-H}^+$. Two other significant peaks were at m/e 729 and 789 corresponding to the previous molecular ions but without their sugar moiety (fig 3).

Collected HPLC fractions corresponding to extracted milk and standards of ivermectin were examined with mass fragmentometry using selected ions at m/e 729, 789, 873, 933. Selected ions profiles obtained with standards of ivermectin agree well with those of extracted milk samples.

Kinetic study

Figure 4 presents the plasma and milk concentration time profile curve of ivermectin after subcutaneous injection in a cow. In plasma, the maximum levels of concentration (20 to 25 ng/ml) was observed during the period 6 to 13 days post-injection and decreased thereafter.

In milk, the peak level (75 ng/ml) was reached 8 days after administration and then decrease slowly according to the long terminal half-life observed in the plasma.

Discussion

Two analytical methods have been described to be used in pharmacokinetic studies. The first (Tway *et al* 1981) is based upon the detection of a fluorescence derivative of ivermectin following high performance liquid chromatography. This method is long and tedious, furthermore the proposed isolation and derivatization steps were unable to use it on milk. The second method (Pivnichny *et al* 1983) which has been used to analyse plasma samples is a direct HPLC proce-

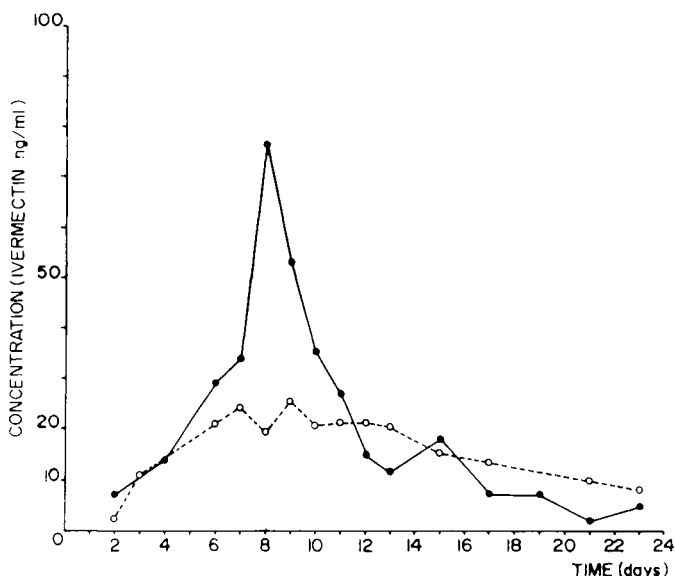


Fig 4. — Plasma (O - - O) and milk (● - ●) time curve in a cow given a 0.2 mg/kg subcutaneous dose of ivermectin.

ture with photometric detection. Due to the extraction procedure this method is not appropriate for milk samples analysis.

In this paper, we developed a sensitive and precise assay for ivermectin in milk with a high degree of specificity confirmed by mass spectrometry. The preliminary results of the pharmacokinetic study confirmed the high persistence of ivermectin in milk. These data are in good agreement with those obtained for both kinetic and residue

study in cattle (Tway *et al* 1981; Schivitzerling *et al* 1985) who revealed high persistence of ivermectin in plasma tissues.

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Abstract

A sensitive and specific high performance liquid chromatographic technique is described for the determination of ivermectin in milk. Milk samples (1 ml) were extracted with organic solvent (iso-octane and acetonitrile) and then chromatographed using reversed phase system. Specificity of the assay was confirmed by the use of mass spectrometry. After an subcutaneous administration of ivermectin (0.2 mg/kg) in a cow, ivermectin was detected in milk for about three weeks and over this period milk and plasma concentration were of the same order of magnitude.

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