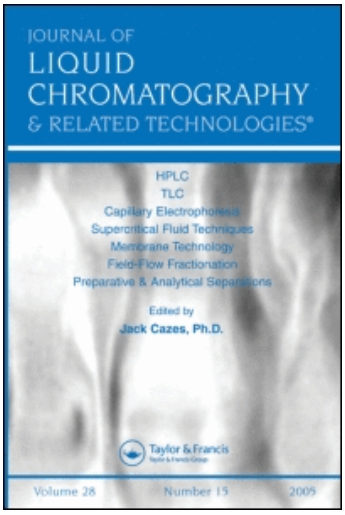

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HPLC DETERMINATION OF RESIDUAL IVERMECTIN IN CATTLE DUNG FOLLOWING SUBCUTANEOUS INJECTION

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ABSTRACT

Residual ivermectin in cattle dung was determined in order to measure its rate of excretion and persistence under field conditions. The drug was extracted into methanol and subsequently determined by reversed-phase high performance liquid chromatography using a 300 × 3.9 mm Bondclone C_{18} column, 47:33:20 acetonitrile/methanol/water as the mobile phase and UV detection at 245 nm. The determination limit for 5 g of sample was 0.020 mg kg^{-1}. HPLC analyses for aminoacids in the dung were suggestive of changes in their contents with time, which may account for the differential behaviour of insects attracted by the dung.

INTRODUCTION

Many different helminthicidal products used to treat animals are eventually found in their excrement (urine and faeces). Some of the products only have a limited action on the coprophagous fauna, but others destroy virtually all insects attracted to the excrement over a more or less long period (1). Products recently introduced to the market have a wide spectrum of activity.

Ivermectin (22,23-dihydroavermectin B_1), one of the most commonly used substances in preventative treatments, is a highly effective systemic antiparasitic against both internal (gastrointestinal and respiratory) parasites and external parasites (flies, ticks) (2). This drug is usually administered via subcutaneous injections in typical doses of 200 $\mu g\ kg^{-1}$ body weight; most of the injected substance is subsequently excreted as such in faeces depending on the particular animal or administration route (3). Ivermectin in faeces preserves its insecticide action, which has fostered studies on its potential environmental impact, *i.e.* its effect on non-target organisms in the soil (5–10). Many such studies were carried out in laboratory conditions and provided results that differed markedly from those obtained under real conditions, as we have recentlky shown the potential impact of the drug depends on the particular region, climate, insect fauna and —probably— year season (11).

Deriving a factual cause–effect correlation entails the accurate measurement of residual amounts of the drug in animal excretions. Initially, this was done by using high performance liquid chromatography (HPLC) with derivatization and subsequent fluorescence detection of the derivative obtained, this derivatization

procedure, based on various reactions, is usually labour-intensive (12–16), so UV detection procedures have also been used , usually at 245 nm (17–23). These procedures have so far been most often applied to such samples as serum, plasma, milk, vegetables and swine tissues, using a prior extraction–cleanup procedure with acetonitrile, methanol/acetonitrile, methanol or ethyl acetate and the aid of sonication or maceration, or, alternatively, solid–liquid extraction cartridges and later the extracts are preferably analyzed on a C_{18} column and with ternary mobile phases ($CH_3CN/CH_3OH/H_2O$).

To extract the drug from the faeces two procedures have been mainly proposed: extraction with acetone (24) or a Soxhlet (6 h) with an ethylacetate/methylene chloride mixture(25).

In this work, a HPLC procedure for the determination of residual ivermectin in cattle dung was developed in order to quantify the drug excretion by cattle and its environmental persistence under field conditions. At a subsequent stage, dung samples were used to determine aminoacids, by using a procedure originally developed for rat faeces (26), in order to study the anomalous behaviour of insects in the field.

EXPERIMENTAL

Reagents

Procaine and aminoacid standards were supplied by Sigma Aldrich Química S.A. (Madrid, Spain). An ivermectin standard and Ivomec^R were supplied by Merck Sharp & Dohme de España S.A. (Madrid, Spain). HPLC gradient-grade methanol

and acetonitrile were purchased from Scharlau (Barcelona, Spain). Finally, nanopure water obtained from a Milli-Q apparatus (Millipore Ibérica, Madrid, Spain) was used throughout.

Apparatus and chromatographic conditions

Ivermectin analysis

The experimental setup used for this purpose was composed of a CM4000 gradient pump, a variable-wavelength SM4000 detector, a CI4000 recorder–integrator and a Dynamixer eluent mixer, all from LDC Analytical (Riviera Beach, FL), in addition to a Marathon Injector from Spark Holland (Emmen, The Netherlands) and a 300 × 9 mm Bondclone 10C18 column from Phenomenex (Torrance, CA). The mobile phase used consisted of 47:33:20 acetonitrile/methanol/water and was passed at a flow-rate of 1 ml/min. The injected volume was 20 µl and degassing was done with helium.

Aminoacid analysis

The assembly used for aminoacid analyses consisted of a mechanical grinder and a Cryodos lyophilizator from Teistar (Terrassa, Barcelona, Spain), a Heidolph tube shaker from Selecta (Barcelona, Spain), and a Pico Tag workstation composed of two 510 pumps, a Satellite WISP autoinjector, a TCH column oven, a UV detector, a System Interface Module and a Maxima Chromatographic workstation, all from Waters Associates (Millipore, Milford, MA). The injected volume used was 8 µl.

Sampling

Faeces were collected from a group of 7 Morucha steers (276 ± 18.6 kg body weight) which had been treated with a subcutaneous injection of ivermectin (Ivomec^{R}) at 200 μg kg^{-1} body weight. Samplings were done at different times (days) after injection. Additional faeces samples were collected from another control group consisting of 100 animals that were given no Ivomec^{R}.

Several experiment series were carried out in order to determine the drug excretion rate and its degradation under the prevailing field conditions.

Samples were divided into portions that were analysed by the three participating workgroups (11). All the samples were stored frozen prior to analysis.

Procedures

Ivermectin analysis

An amount of 5 g of thawed sample was added 25 ml of methanol and the mixture was stirred for 25 min, after which it was centrifuged at 1500 G for 15 min. The supernatant was then concentrated to 7 ml in a rotary evaporator at 80°C and centrifuged under the same conditions as the initial mixture. The resulting extract was added the internal standard at a concentration of 50 ppm and the mixture was made to 10 ml with methanol and filtered through PTFE (Ø = 13 mm, 0.50 μm mesh). Aliquots of 20 μl of the filtrate were used for injection into the chromatograph.

Aminoacid analyses

An amount of 8 g of frozen dung was lyophilized. The lyophilizate was then ground and a portion of 50 mg was weighed and placed in a Corning tube that was introduced into the Pico Tag workstation, where it was subjected to hydrolysis, derivatization and chromatographic analysis.

RESULTS AND DISCUSSION

Extraction of ivermectin from cattle dung

Experiments were initially carried out by using raw dung and then dung + ivermectin and afterwards dung + Ivomec^{R}. Water, water/methanol, methanol and the mobile phase were assayed as extractants and the influence of the solvent volume and extraction time and temperature was studied in order to develop a straightforward, expeditious extraction procedure for the samples.

All the solvents tested gave rise to a common front at 4 min in the experiments involving raw dung. Methanol gave an additional, small peak at 5.80 min which increased in size with the increasing alcohol content in water/methanol mixtures. The mobile phase also gave a further peak at 8.00 min (Fig. 1). Because such peaks would make the chromatogram background and pure ivermectin eluted at 12 min, an internal standard not affected by the background was required. Several compounds were assayed and procaine, which eluted at 7 min, was chosen for this purpose.

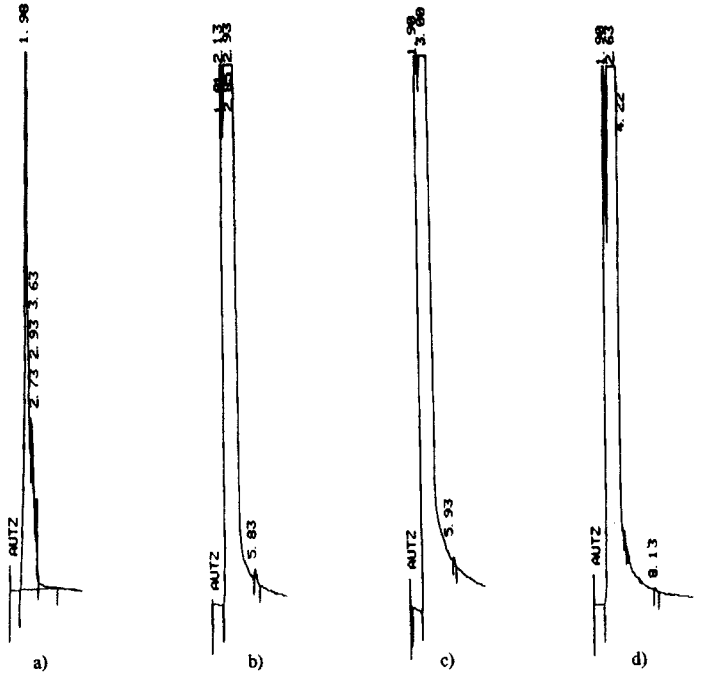

Figure 1.- Chromatograms obtained from raw samples extracted with: a) water, b) methanol, c) water-methanol mixtures, d) mobile phase.

As the analyses of dung samples fortified with ivermectin and Ivomec^{R} provided identical results, in the next paragraphs are described in a common way.

Variable water volumes (10–40 ml) used at different temperatures (20–100°C) and shaking times (10–40 min) provided chromatographic backgrounds that were similar to that described above, the peak for ivermectin did not appear. This suggests that water does not leach ivermectin from dung.

Adding methanol to the water allowed some of the drug to be extracted. However, the extraction yield never exceded 60% at any methanol/water ratio

—the effect of the alcohol content in the mixture was much more marked than that of the time, temperature or extractant volume used.

By using methanol or the mobile phase as extractant, drug recoveries over 90% were obtained at room temperature.

Figure 2 summarizes the results provided by the different solvents that extracted the ivermectin , of which methanol was finally chosen in order to reduce analytical costs and avoid the appearance of the above-mentioned small peak at 8 min. The nature of the matrix implies two centrifugation steps in order to remove solid residues.

Quantitative analysis

Application of the proposed procedure to the cattle dung samples provided chromatograms such as that shown in Fig. 3.

The calibration graph run from fortified dung samples was linear and fitted the equation

$$(A_i/A_s)C_s = 4.09\ C_i - 0.126$$

where A_i and A_s denote the peak areas for ivermectin and procaine, C_s the internal standard concentration and C_i the ivermectin concentration. The variance was 3.680×10^{-4}.

The detection and determination limits obtained for 5 g of sample were 0.010 and 0.020 mg kg^{-1} dung, respectively.

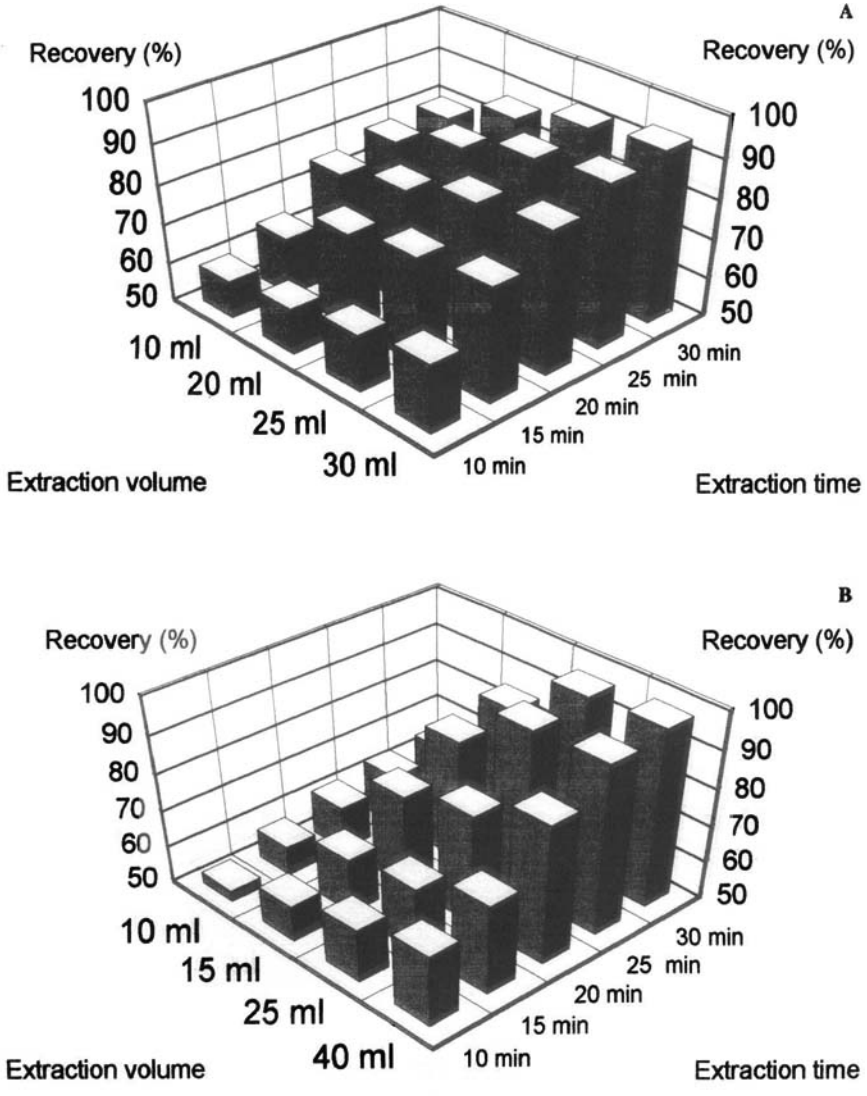

Figure 2.- Ivermectin recovery % vs shaking time and extraction volume with: a) methanol, b) mobile phase, c) 75% water-25% methanol, d) 50% water-50% methanol e) 25% water-75% methanol.

(continued)

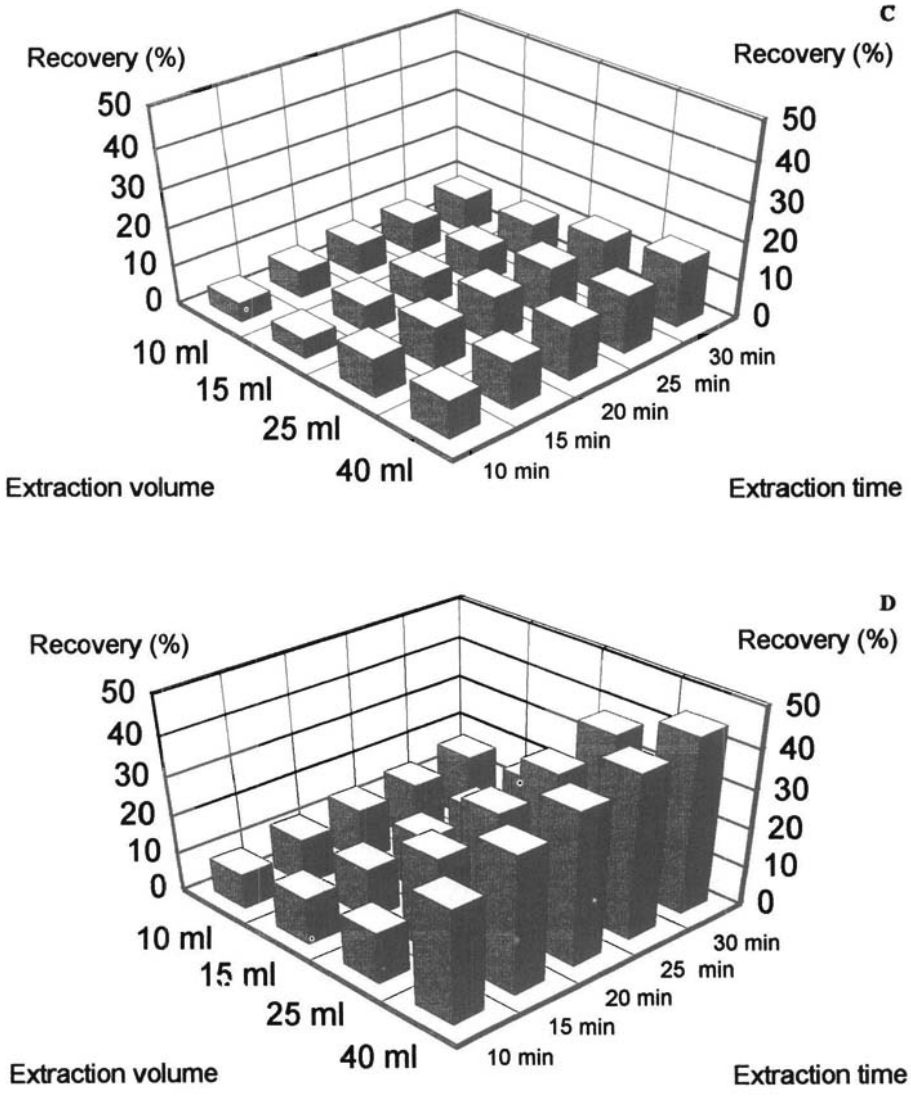

Figure 2 *(continued).*

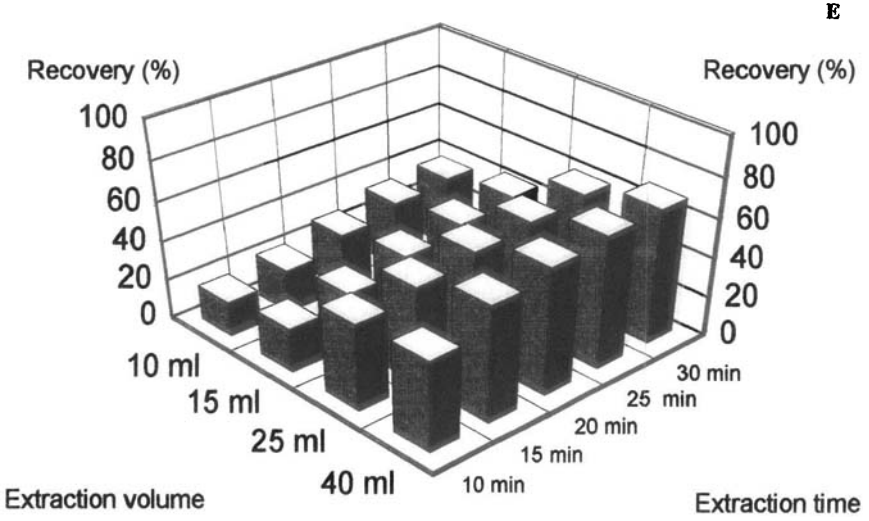

Figure 2 *(continued)*.

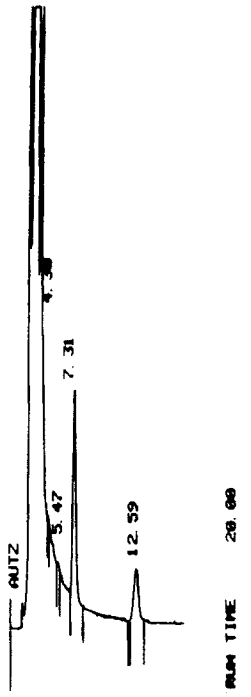

Figure 3.- Chromatogram of a sample fortified with internal standar.

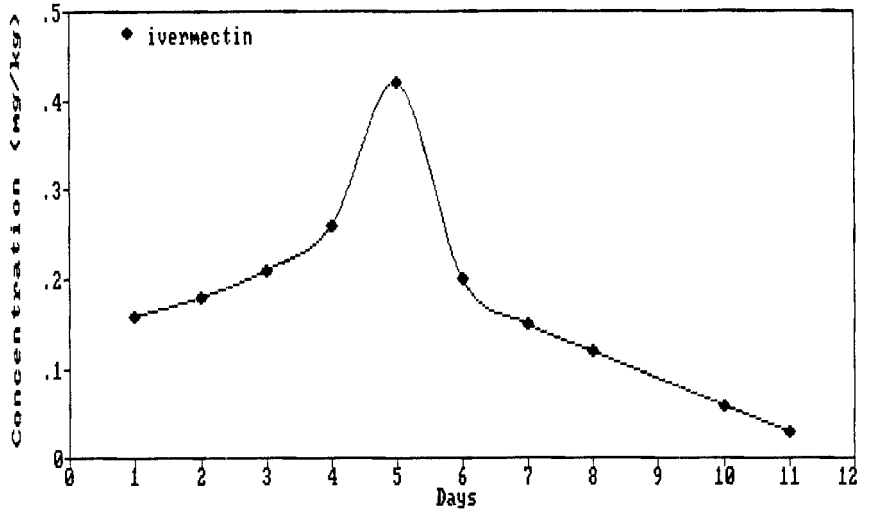

Figure 4.- Ivermectin excretion profile in cattle dung after injection.

Excretion of ivermectin by cattle

Figure 4 shows the variation of the amount of ivermectin found in the dung with time. As can be seen, such an amount peaked *ca.* 5 days after injection and decreased sharply afterwards to levels below the detection limit at 12 days.

A series of dung samples were exposed to field conditions for up to 30 days; none of them was found to contain any ivermectin after 6–7 days; however, the absence of the drug was followed by an increased attractiont of beetles, which were collected from pitfall traps baited with dung. These results (11) suggest that some biochemical process have led to *in situ* modifications in the composition of dung from treated animals,resulting in increasing attractiveness, as reflected in the fetid odour of the dung, similar to that of faeces from omnivores. This led us to

analyse the dung for aminoacids during the exposure period in order to detect any differences in the dung composition before and after treatment.

Aminoacid analyses

As can be seen from Fig. 5, the aminoacid profiles for the treated and untreated samples were indeed different. Analysis on samples previously subjected to ivermectin determinations also revealed the aminoacid contents to change with time after injection. The changes, however, varied from compound to compound. Thus, the concentrations of glutamic and aspartic acid rose up till the fourth day, and later they dropped abruptly. On the other hand, the histidine and methionine concentrations increased slightly for 7 days and then decreased gradually. The alanine, valine and leucine give the highest peaks in the fourth and seventh day, whereas that proline increased throughout the period studied and the other aminoacids assayed (serine, arginine, threonine, tyrosine, isoleucine, phenylalanine and lysine) remained essentially constant.

CONCLUSIONS

Extraction of ivermectin with ethanol is a straightforward procedure that provides drug recoveries close to 100%.

Ivermectin is rapidly excreted by cattle. Its concentration in dung increased daily in pats dropped on days one to four after injection. A peak of elimination was observed for day 5 followed by a quick decrease. After the twelfth day, the drug concentration is below the detection limit of the used procedure.

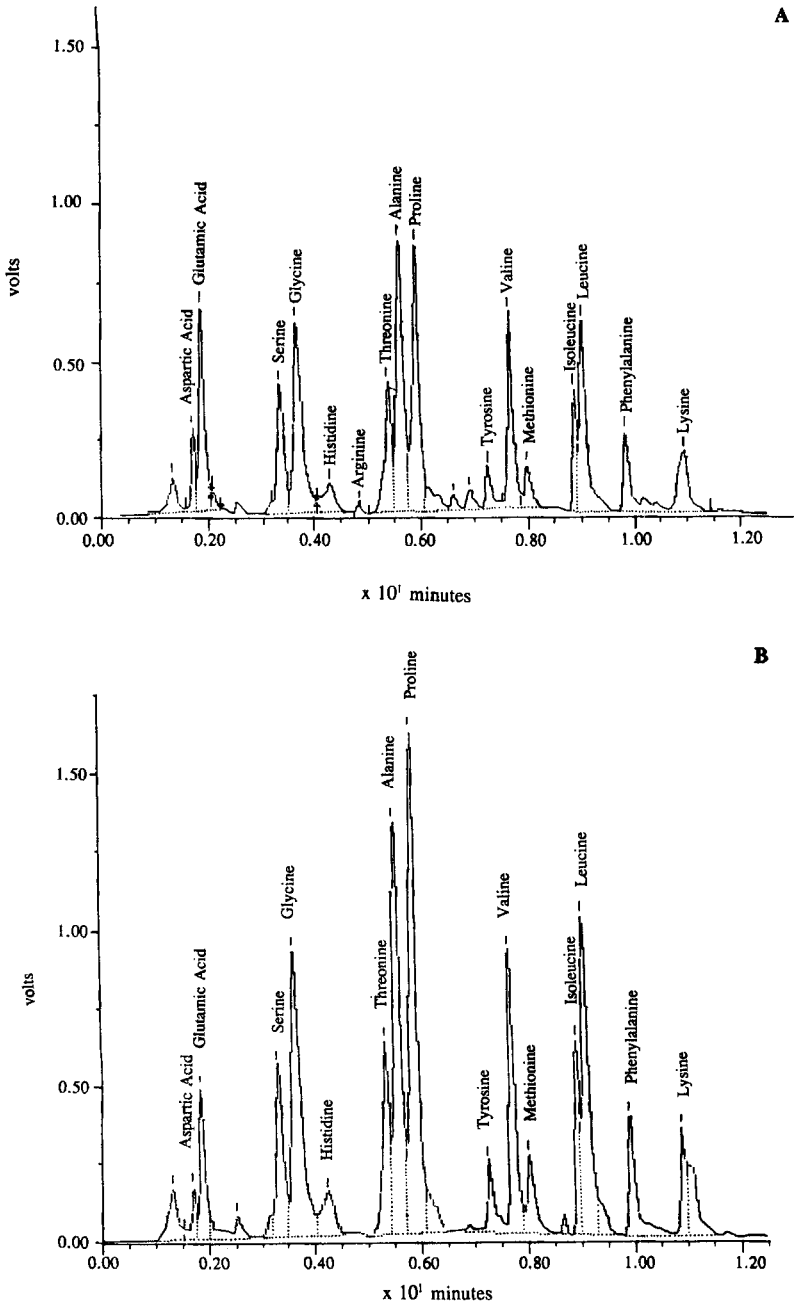

Figure 5.- Chromatograms showing the aminoacids found in cattle dung before (a) and after (b) drug administration.

The residual concentration of ivermectin in dung exposed to field conditions decreases rapidly to undetectable levels after 6 days, when the dung whitens and develops a fetid odour. Also, the aminoacid concentrations decrease after 6–7 days, which might explain the anomalous attraction of the beetles.

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