Journal of Chromatography, 168 (1979) 179–185

CHROM. 11,280

ELECTRON-CAPTURE DETECTION OF CHLORAMPHENICOL USING A HEPTAFLUOROBUTYRATE DERIVATIVE

APPLICATION TO RESIDUES IN MILK

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(Received June 6th, 1978)

SUMMARY

A gas–liquid chromatographic method is described for the quantitation of the halogenated heptafluorobutyrate derivative of chloramphenicol, using electron-capture detection. Treatment of the chromatographic support with heptafluorobutyric anhydride permits a linear recovery even at very low concentrations. After extraction using acetonitrile followed by a convenient cleaning procedure, this method can be applied to the determination of trace amounts of chloramphenicol in milk. Interfering peaks have been observed on the chromatograms of some control milk samples. These determine the detection limit of 50 ppb.

INTRODUCTION

Chloramphenicol (CP) is a valuable and widely used antibiotic for the treatment of gram negative infections in cattle, specially mastitis in cows. However, several workers have demonstrated the persistence of CP residues in milk. The passage of CP from blood to milk has been demonstrated after parenteral injection, as well as a gradual elimination in milk after intra-mammary administration[1,2]. Moreover, the use of CP has been criticized for causing bone-marrow damage in humans, and zero tolerance levels have been proposed for CP in edible tissues in order to protect the consumer against these toxic effects. Until now the lack of a sensitive and specific analytical method has precluded the detection of very low concentrations of CP.

Bio-assays are most often used[3], but their sensitivity is limited (0.5–1 ppm) and the precision appears to be variable; moreover, their specificity is poor in samples containing other antimicrobial agents, such as are present in milk. Colorimetric assays have also been developed[4]. Each of these measures the amine formed after reduction of the aromatic nitro group of CP, then all the CP derivatives and metabolites containing this function are determined at the same time. This lack of specificity leads

to values 50–60% higher than those obtained microbiologically[1]. Even when associated with a specific solvent extraction of CP, the sensitivity is limited to 0.5–1 ppm.

More recently, gas–liquid chromatographic (GLC) methods have been proposed, based on derivatization of the CP molecule in order to obtain a volatile compound. Trimethylsilyl ethers[5,6] and acetate[7] have been shown to be suitable derivatives, enabling a more specific and sensitive determination. About 5 ng of CP may be detected using a flame ionization detector. However, taking into account this intrinsic response, the lowest detectable concentration in biological fluids is dependent on the size of the sample and on the level of interfering substances it contains. The methods involving trimethylsilyl ether derivatives were proposed for rapid routine clinical investigations in sera of patients treated with CP; the simplified cleaning procedure generally adopted, and the small size of the sera samples, reduced the detection levels to 0.5 ppm. The method involving the acetate derivative was carried out in order to detect trace amounts of CP in agricultural crops; a detection limit of 80 ppb was achieved using 100-g samples, but an extensive cleaning procedure was necessary.

Our aim was to lower the detection levels of conventional methods by a factor of 10, using a simpler procedure and reasonably sized milk samples (10 ml). A study of the possibilities offered by the very sensitive electron-capture detector have led to the method proposed, which involves the preparation of a halogenated heptafluorobutyrate derivative.

MATERIALS AND METHODS

Reagents

Acetonitrile, isooctane (2,2,4-trimethylpentane) and ethyl acetate, all analytical grade (Prolabo, Paris, France), were distilled over a 1-m adiabatic column before use. Heptafluorobutyric anhydride (HFBA) was supplied by Fluka (Buchs, Switzerland), and analytical grade chloramphenicol by Calbiochem (Los Angeles, Calif., U.S.A.). [^{14}C]Chloramphenicol (dichloro [1-^{14}C]acetyl, D-*threo* form) was obtained from the Radiochemical Centre (Amersham, Great Britain).

Apparatus

A Girdel 3000 gas chromatograph equipped with an electron-capture detector (^{63}Ni source) operated in the pulse mode (pulse withd, 0.8 μsec; pulse period, 200 μsec; pulse amplitude 10 V) was employed. The glass column (1.50 m × 2 mm I.D.) was packed with 3% Dexsil 300 on Supelcoport AW DMCS (100–120 mesh) (Supelco, Bellefonte, Pa., U.S.A.); nitrogen flow-rate, 20 ml/min; oven temperature (isothermal), 195°; injector temperature, 220°; detector temperature, 290°. Two successive injections of 5 μl HFBA once a week (detector disconnected) were followed by conditioning for 24 h at 250° under a stream of nitrogen.

Procedure

A 10-ml sample of milk was placed in a 125-ml PTFE-stoppered glass bottle, Forty millilitres acetonitrile were added, the bottle was shaken vigorously, and the contents left to settle. The mixture was then filtered through glass wool into a 250-ml separating funnel and the flask was rinsed with another 10 ml of acetonitrile. A 25-ml

volume of isooctane was added, the mixture shaken for 2 min and then left to settle again for 30 min. The acetonitrile phase was collected in a 250-ml round-bottomed flask and evaporated at 40° *in vacuo*.

The residue was dissolved in 10 ml of distilled water and transferred to a 125-ml separating funnel; 1 ml of phosphate buffer (pH 6) was added and the aqueous phase extracted twice with 25 ml of isooctane. The organic phase was discarded. A 0.5-ml volume of 1 *N* sodium hydroxide was added to the aqueous phase which was then extracted twice with 25 ml of issooctane. The organic phase was again discarded. The aqueous phase was adjusted to pH 6.5 by adding 1 % acetic acid, then extracted twice with 25 ml of ethyl acetate. The organic phase was washed with 10 ml water, filtered on anhydrous sodium sulphate, evaporated partly *in vacuo*, transferred to a 10-ml PTFE-stoppered conical flask and then evaporated to dryness under a stream of nitrogen.

A 100-μl volume of HFBA was placed in the conical flask, which was stoppered tightly and placed in an oven set at 75°, for 30 min with intermittent shaking. After cooling, the non-reacted anhydride was evaporated under a stream of nitrogen and the residue dissolved in 5 ml of isooctane. Two millilitres of distilled water were added, followed by vigorous shaking. After partition, 2 μl of the organic phase were injected into the chromatograph within 10 min of the last purification.

Quantitation was performed using derivatized standard CP as a reference. Two microlitres of a dilute solution, corresponding to the same range of CP concentration as in the samples, were injected after every five samples to correct for variations in the detector response.

RESULTS AND DISCUSSION

Derivatization

Theoretically, three derivatives may be obtained by reaction of HFBA with the primary hydroxyl, secondary hydroxyl or both these functions of the CP molecule. Different reaction conditions were applied, and the final products were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Fig. 1 indicates that, under mild conditions, *i.e.*, at laboratory temperature, three peaks (I–III) were obtained, while only one (I) appeared when heating at 75° for 30 min. On a silicone phase such as Dexsil, the elution order reflects first the volatility of the substances; thus the peak having the lowest retention time (I) corresponds to the diester. The structure of compound I has been confirmed by GC–MS analysis, showing the molecular peak $M^+ = 715$ and the main fragments indicated in Fig. 2.

Taking into account the polarities of the different derivatives, the elution order of the mono-heptafluorobutyrates ought to be as follows: first the esterified primary hydroxyl group (peak II), then the esterified secondary hydroxyl group (III).

The formation of only the diester derivative as a single and major peak (I) gave an easier and more sensitive determination of CP. A reaction time of 30 min at 75° proved to be the most satisfactory condition for a complete esterification of the molecule.

Limit of detection

The electron-capture detector is not very sensitive to CP in spite of the presence

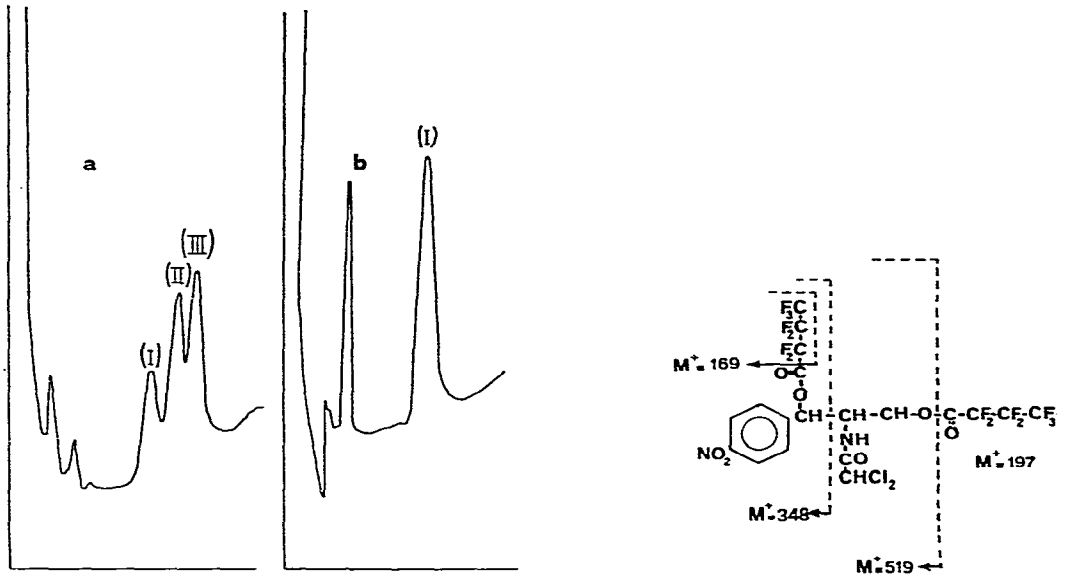

Fig. 1. Gas chromatograms of CP HFBA derivatives formed at room temperature (a) and at 75° for 30 min (b). Peaks: I = diester derivative; II = monoester on primary hydroxyl; III = monoester on secondary hydroxyl.

Fig. 2. Mass spectrometric fragmentation and structure of compound I.

of two chlorine atoms and the electronegative NO_2 group. However, when the CP molecule is enriched with halogen the resulting derivative is highly reactive. The detection limit is dependent both on the quantitative derivatization of microquantities, and on the absence of interactions between the chromatographic support and the derivative.

The first point was examined using [^{14}C]CP. Ten, 1 and 0.1 μg quantities of labelled CP were treated with 100 μl of HFBA, and the derivative was separated from residual unchanged CP by partition with water–isooctane. Esterification was complete (92% recovery) in every case.

It is well known that the active hydroxyl groups of silica type GC supports may form hydrogen bonds with chromatographed substances and affect the quantitative recoveries at low concentrations. Different column treatments have been proposed in order to reduce these losses. Fig. 3 indicates that this phenomenon occurs to a marked extent with CP heptafluorobutyrate, even with a dimethylchlorosilane-treated support. Accordingly, we attempted to block the free remaining OH sites by the same reagent involved in the derivatization process, *i.e.*, HFBA. The result was an original and very effective treatment (see Materials and methods), that increased the range of the linear response toward lower concentrations.

A chromatogram obtained under such conditions with standard CP (Fig. 4) shows a peak with a retention time of 3 min. A 25% full scale recorder response was observed for 40 pg of CP injected in the derivatized form.

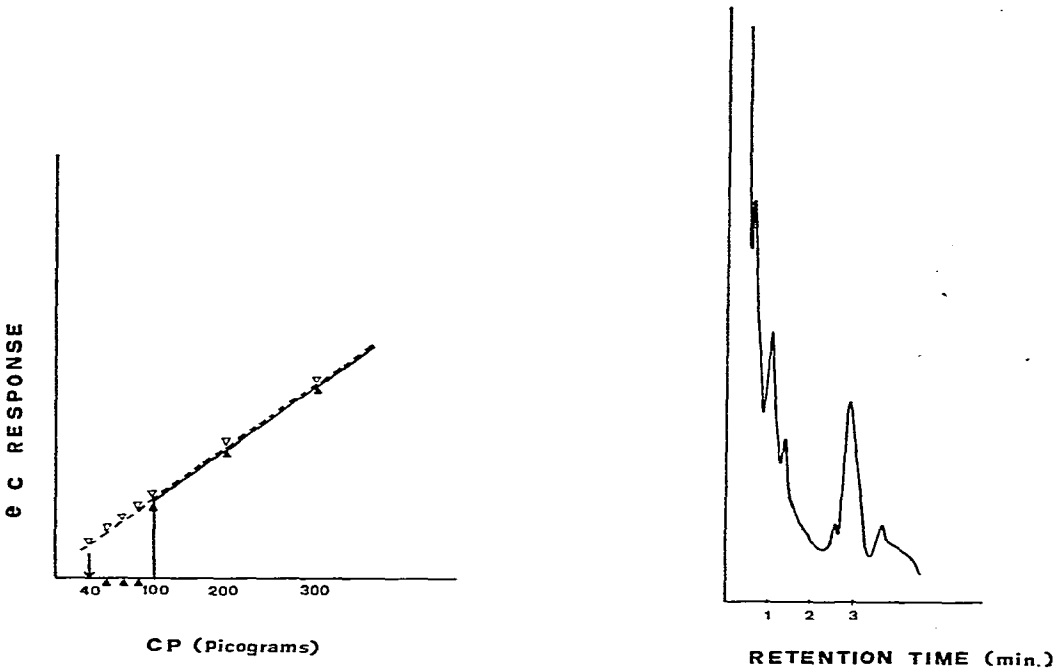

Fig. 3. Effect of HFBA treatment of the chromatographic support on the recovery of micro-quantities of CP injected as the HFBA derivative. ▲—▲, Silanized commercial support; △- - -△, HFBA-treated support.

Fig. 4. Gas chromatogram of standard CP–HFBA derivative. Amount derivatized 0.1 μg CP; injected, 40 pg of derivatized CP.

Determination in milk

Fig. 5 summarizes the different steps of extraction, cleaning procedure, derivatization and analysis. CP is generally extracted from biological samples, using ethyl acetate or isoamyl acetate[5,6]. However, poor partition ratios occur with milk due to hindering emulsions. This difficulty has been overcome by using acetonitrile, which is a very good solvent for CP, and also precipitates proteins which makes the subsequent cleaning procedures easier. Milk lipids are extracted with isooctane. Most remaining interfering substances are eliminated by the liquid–liquid partitions at acidic and basic pH values successively.

The different stages of the analytical procedure, *i.e.*, extraction, cleaning and derivatization, were tested separately for CP recovery, using [^{14}C]CP as a tracer. The influence of accompanying substances on the yield of the derivatization was particularly investigated. The results (Fig. 5) indicate that derivatization is almost complete, and overall recovery good (80.9%). Under these conditions, the results obtained with standard CP should permit a detection limit of *ca.* 10 ppb in milk.

However, this limit of detection is dependent on the importance of interfering peaks that appear at the same retention time on control sample chromatograms (Fig. 6). Ten successive determinations carried out on different control milk samples showed interfering peaks whose average area was equivalent to 100 pg of CP injected in the

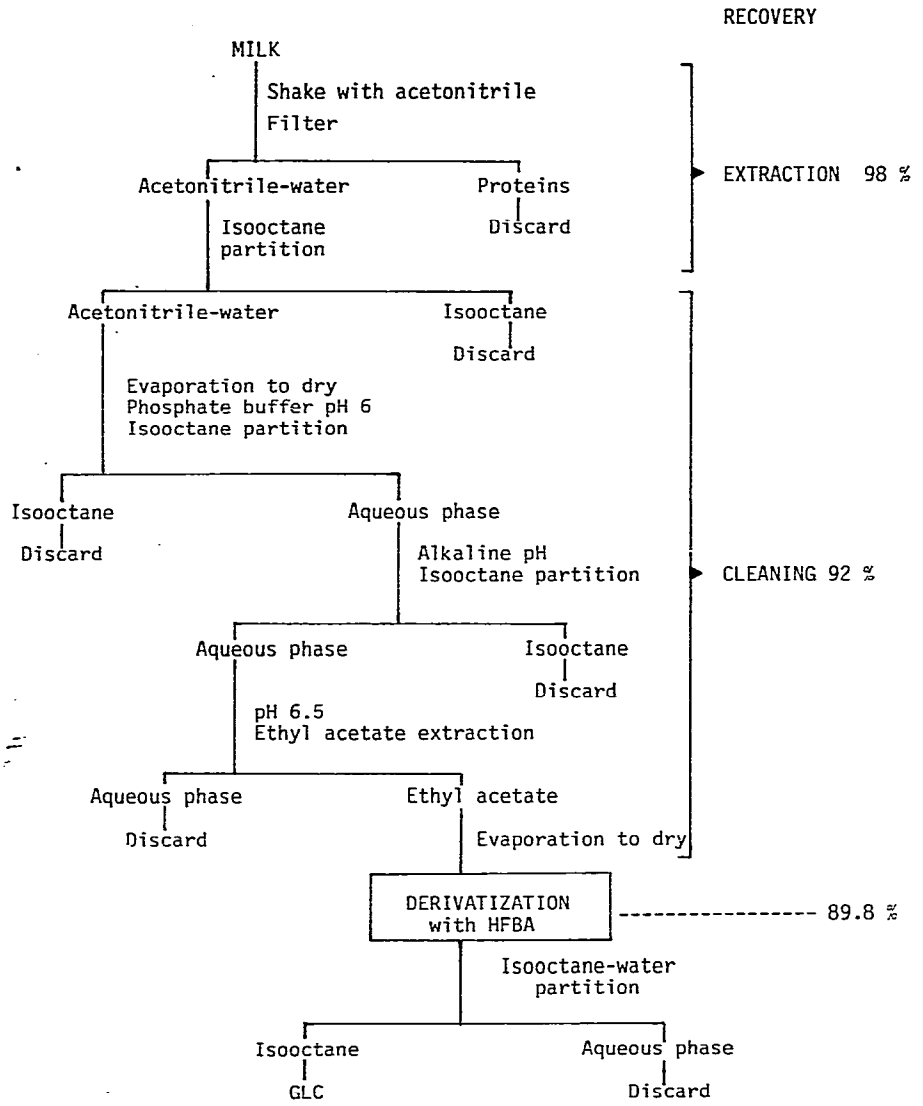

Fig. 5. Summary of the complete analytical procedure. The recovery was determined for each of the main steps using [^{14}C]CP.

HFBA derivative form. The coefficient of variation measured was 36%. These results, expressed as the CP concentration in milk, would correspond to 25 ± 9 ppb. The variability for control milks indicates that peaks observed in the chromatograms of unknown samples may be significant for CP when the responses are three standard deviations larger than this average concentration. The confidence limit is then better than 99%. Thus, this method permits a quantitative determination of CP in milk in concentrations as low as 50 ppb.

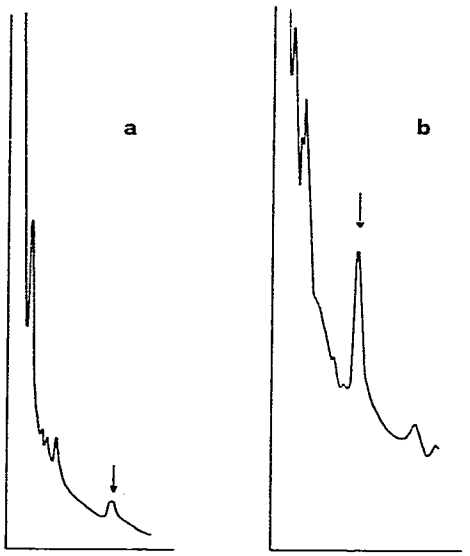

Fig. 6. Gas chromatograms obtained from samples of control milk (a) and the same fortified with 0.1 μg of CP per ml (b).

CONCLUSION

The GLC analysis described combines HFBA derivatization of the CP molecule with HFBA treatments of the chromatographic support in order to avoid non-specific absorption at low levels. It allows a very high sensitivity of detection (40 pg), and can be applied to biological fluids, especially milk, with a cleaning procedure that ensures good recovery. However, in spite of the specificity of the GLC and electron-capture detection, interfering peaks having the same retention time as CP are observed for some control samples. These substances constitute the only limitation on decreasing the sensitivity of detection. Taking into account the variability of such interferences, 50 ppb of CP in milk is a concentration that can be detected with a statistically high confidence level.

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