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Note

Derivatization–gas chromatographic analysis of cinoxacin in capsules as its pentafluorobenzyl derivative

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Cinoxacin (CX, 1-ethyl-1,4-dihydro-4-oxo[4,5-*g*]cinnoline-3-carboxylic acid) is a synthetic antibacterial agent[1–3] used mainly for the treatment of urinary tract infections, caused by susceptible gram-negative bacteria.

Several methods, including non-specific bioassay[4–7], semi-quantitative thin-layer chromatography[8], fluorometry[9], and high-performance liquid chromatography[10,11] with UV detection without prior derivatization have been developed for the analyses of cinoxacin and its metabolite in various specimens. However, a literature survey indicated that there is no gas chromatographic (GC) method for the determination of CX except the one[12] established by us for the determination of CX as its methyl ester based on derivatization using diazomethane, which is generally supposed to have both toxic and explosive potential.

This paper presents a simple GC method based on analytical derivatization of CX by pentafluorobenzyl bromide (PFBBr). The method was satisfactorily applied to the analysis of CX in capsules by GC with flame-ionization detection (FID). Simultaneous resolution of CX from closely related compounds such as nalidixic acid, oxolinic acid and piromidic acid is feasible by chromatography with temperature-programming.

The method should be suitable for the trace analysis of CX by GC with electron-capture detection (ECD), because the derivative of CX contains a pentafluorophenyl moiety, which is highly sensitive to ECD.

EXPERIMENTAL

GC conditions

A Varian 1740 gas chromatograph equipped with a flame ionization detector was used. The column was a 90 cm × 2 mm I.D. coiled glass tube packed with 2% OV-1 on Chromosorb W AW DMCS (80–100 mesh). The injection port and detector temperatures were kept at 290°C and 295°C, respectively. The column temperature was isothermally set at 255°C, unless indicated otherwise. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min. A PTFE-faced septum (Varian Aerograph) was used in the injection port. Column pretreatment by repeated injections of a suitable amount of the CX derivative solution is essential to prevent column loss, which occurred in the initial chromatographic analysis.

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Mass spectrometry

A JEOL JMS-D-100 mass spectrometer was employed with an ionization source temperature of 200°C, an electron energy of 75 eV and an acceleration energy of 3 kV.

Chemicals and reagents

CX (a gift from Eli Lilly, IN, U.S.A.), *n*-tetratriacontane (guaranteed grade; Tokyo Kasei, Tokyo, Japan), 2% OV-1 on Chromosorb W AW DMCS (80–100 mesh) (Wako, Osaka, Japan), α-bromo-2,3,4,5,6-pentafluorotoluene (PFBBr) (Aldrich, Milwaukee, WI, U.S.A.), potassium carbonate, acetonitrile, dichloromethane and other reagents were of analytical-reagent grade.

Solutions of the internal and reference standard were separately prepared: by accurately weighing *ca.* 25 mg of *n*-tetratriacontane into a 100-ml volumetric flask and dissolving and diluting to volume with dichloromethane, or *ca.* 100 mg of CX into a 50-ml volumetric flask and dissolving and diluting to volume with acetonitrile. Further dilution was made if necessary.

Sample preparation

The content of each capsule was accurately weighed, and a suitable amount equivalent to *ca.* 50 mg of CX was transferred to a 25-ml volumetric flask. The volume was made up with acetonitrile, and the mixture was stirred for 30 min by means of a magnetic stirrer. A 0.5-ml aliquot of the sample solution was pipetted and derivatized.

Derivatization procedure

A 0.5-ml volume of the reference standard or sample solution was placed in a 10-ml glass-stoppered test tube containing *ca.* 150 mg of potassium carbonate, and 7 μl of PFBBr were added. The reaction mixture was mechanically shaken for 1 h at 70°C. After cooling, 1.0 ml of the internal standard (I.S.) solution was introduced into the reaction solution, and an aliquot of it was subjected to GC analysis. A calibration graph was constructed by plotting the amount of CX *versus* the peak-height ratio of the CX derivative to the I.S., and the CX content in capsules was calculated from the calibration graph obtained. The peak-height ratio of the CX derivative to the I.S. was also used to evaluate conditions for optimization.

RESULTS AND DISCUSSION

Direct GC determination of CX is unsuitable, because CX is a highly polar compound and decomposes around its melting temperature[13]. Therefore, chemical modification of CX is required to improve its volatility and stability for better chromatoghraphic properties. Analytical derivatization of CX at its carboxyl function with PFBBr was tried, and several parameters affecting the derivatization were investigated to establish the optimum conditions for pentafluorobenzylation of CX (3.8 μmol used for evaluation).

Investigations of the effects of the reaction temperatures and reaction time indicate that formation of the CX derivative reaches a plateau after reaction at 70°C for 1 h in good yield, but at 30°C the reaction takes more than 2 h to reach equilibrium, and then in much lower yield.

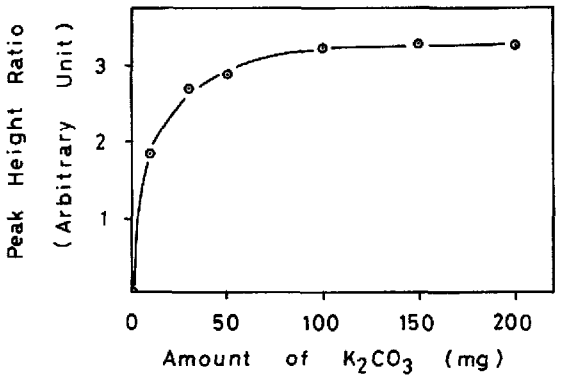


Fig. 1. Effect of amount of potassium carbonate on the formation of cinoxacin derivative; each point indicates the average of three measurements.

The organic solvens acetonitrile, acetone and dichloromethane were tested for derivatization, and acetonitrile was found to be the best as evaluated by the method described for the derivatization procedure, except that the reaction temperature was kept at 30°C to prevent these solvents from boiling. Therefore, acetonitrile was selected as the reaction solvent.

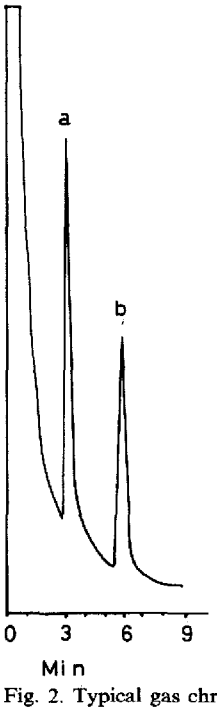


Fig. 2. Typical gas chromatogram of cinoxacin as its pentafluorobenzyl derivative. Peaks: a = the derivative of cinoxacin; b = *n*-tetratriacontane (internal standard). Conditions as in the text; sample injected, 3 μl.

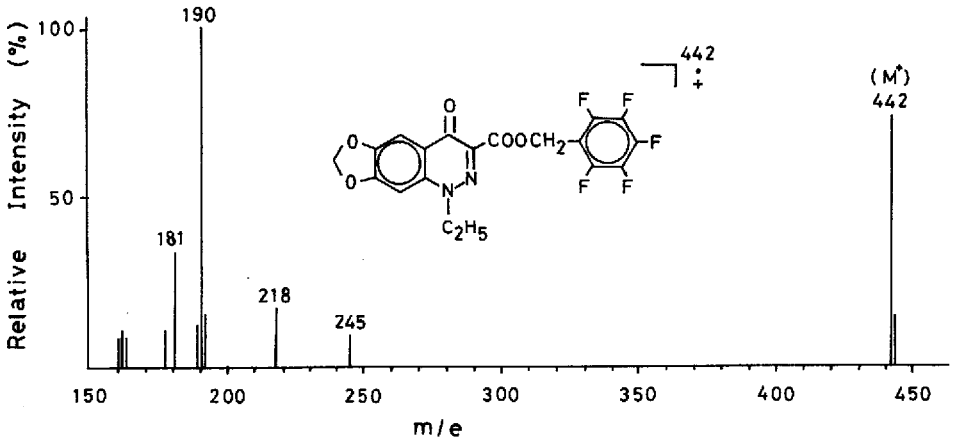


Fig. 3. Mass spectrum of the pentafluorobenzyl ester of cinoxacin. Conditions as in the text.

The amount of potassium carbonate (30–50 mesh) required as the base-catalyst for derivatizaton of CX is *ca.* 100 mg (Fig. 1). Derivatization in a system without potassium carbonate results in a negligible amount of the CX derivative.

The amount of PFBBr required for optimum derivatization of 3.8 μmol of CX is *ca.* 5 μl, and 7 μl of PFBBr were used, equivalent to a molar ratio of 12 to the amount of CX.

The quantitative applicability of the method was evaluated by analysing seven different amounts of CX over the range *ca.* 0.3–3.8 μmol. A linear regression equation ($y = 3.479x - 0.197$) was obtained with a correlation coefficient of 0.999, indicating the good linearity of the method. The recovery of CX was tested on known amounts of samples containing six different levels of CX. The overall recovery for the six samples was 100.8 ± 3.4% (standard deviation), which shows that the precision of the method is satisfactory. The derivatization yield of CX was *ca.* 94%, based on the

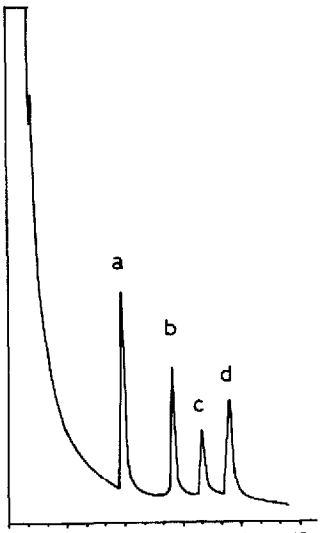
TABLE I

ASSAY RESULTS FOR CINOXACIN OBTAINED FROM A COMMERCIAL SOURCE

Each capsule contained 500 mg of CX.

Capsule	*Amount found* (mg per capsule)*	*Recovery (%)*
C1	507.7	101.5
C2	494.0	98.8
C3	475.5	95.1
C4	498.4	99.7
C5	516.5	103.3
C6	494.5	98.9
	Mean recovery (%)	99.6
	S.D. (%)	2.8

* Average of five replicate determinations.



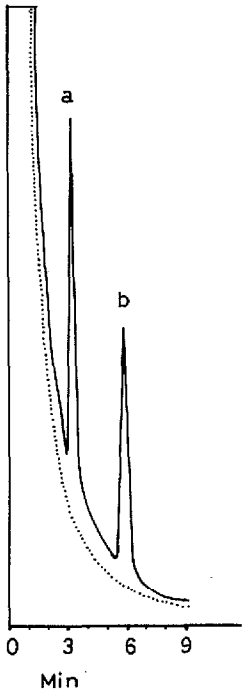


Fig. 4. Gas chromatogram from a mixture of micromolar levels of urinary anti-infectives per millilitre of solution derivatized with PFBBr. Peaks: a = nalidixic acid; b = cinoxacin; c = oxolinic acid; d = piromidic acid. Conditions: glass column, 90 cm × 2 mm I.D., 2% OV-1 on Chromosorb W (80–100 mesh); nitrogen flow-rate, 30 ml/min; column temperature, initial at 210°C for 3 min after injection and programmed at 10°C/min to 270°C, then held for 3 min; sample injected, 4 μl; flame ionization detection.

Fig. 5. Composite gas chromatogram of urine blank from an extract of 3 ml of normal urine (dotted line) and the urine spiked with CX (solid line). Peaks: a = the derivative of cinoxacin; b = *n*-tetratriacontane (internal standard). Sample injected, 3 μl; further details as in the text.

calculation of the peak-height ratio of a reference standard solution containing 3.8 μmol of CX to I.S. in comparison with that of the CX derivative synthesized. Fig. 2 shows a typical gas chromatogram of the CX derivative with a short retention time. Better resolution of the peak of the CX derivative from that of the tailing solvent can be obtained by the following GC conditions with temperature-programming: the column temperature was held at 245°C for 1 min after injection and then programmed at 10°C/min to 280°C, then held at that temperature for 3 min. But for simple analysis the column was isothermally operated at 255°C. The retention time of peak a in Fig. 2 is identical with that of the pentafluorobenzyl ester of CX, synthesized by modifying the derivatization procedure. The crystal purified was subjected to mass spectrometric analysis, and the spectrum obtained exhibited a parent ion at $m/e = 442$, corresponding to the pentafluorobenzyl ester of CX as indicated in Fig. 3.

The method was applied to the analysis of CX in capsules and the results are presented in Table I. The method with the temperature-programming technique was also tried in the analysis of a sample mixture of urinary anti-infectives of closely related structures, including CX, nalidixic acid, oxolinic acid and piromidic acid. Fig.

4 shows that the structurally related compounds were well resolved, and indicates that individual determination or simultaneous determination of these compounds could be expected if the method were suitably modified.

Preliminary tests on the extraction of CX spiked in a normal urine were performed. As shown in Fig. 5, no components in the urine blank interfered with either the CX derivative or the I.S. added in the urine. Therefore, the application of the method to analysis of CX in urine seems to be possible. The brief procedure for the extraction of CX in urine is as follows: to 3.0 ml of urine spiked with CX (0.5 m*M*), prepared by dissolving the acidic CX in urine adjusted to pH 9.0, 0.5 ml of hydrochloride acid (0.5 *M*) was added. Then the acidified urine solution was extracted with 5.0 ml of chloroform. An aliquot of 3.0 ml of the separated chloroform extract was subjected to evaporation at 60°C under nitrogen. The residue obtained was dissolved with 0.5 ml of acetonitrile and then derivatized as described under the derivatization procedure. GC analysis was executed by injection of 3.0 μl of the derivatized solution.

Further evaluation of the method for the analysis of CX in biological specimens, and possible modification of the method for the determination of other urinary anti-infectives, are under investigation.

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