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IMPROVED GAS CHROMATOGRAPHIC METHOD OF DETERMINING DICLOFENAC IN PLASMA

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SUMMARY

Diclofenac was converted into either its methyl or ethyl ester in methanol or ethanol containing 0.1% or 0.5% sulfuric acid, respectively. The ester was extracted and subjected to gas-liquid chromatography with electron-capture detection. The esterification resulted in an increased sensitivity of the gas chromatographic detection, three times better than that previously reported for the formation of indolone ring in trifluoroethanol containing 0.5% sulfuric acid.

INTRODUCTION

Diclofenac [sodium *o*-(2,6-dichlorophenyl)-aminophenylacetate; the active constituent of Voltaren] is pharmacologically active as an anti-inflammatory and antirheumatic agent. Two methods for assaying diclofenac in biological materials have been reported [1, 2]. Both methods are based on indolone ring formation: one in trifluoroethanol containing 0.5% sulfuric acid [1], the other in methanol saturated with HCl gas [2]. We applied both methods to the determination of diclofenac in rabbit plasma after drug administration. The second method did not give good reproducibility because of interference in the reaction by concomitant moisture. In the first method, however, the presence of methanol in the trifluoroethanol gave a sensitivity about three times higher than that previously reported. For convenience, we routinely used the methanol-trifluoroethanol method for the quantitation of diclofenac. Meanwhile, using thin-layer chromatography and gas chromatography-mass spectrometry

(GC-MS), we identified a GC peak with a retention time of 16 min as the indole derivative and the methyl ester of diclofenac. On the basis of these observations and the fact that trifluoroethanol is a relatively expensive material, we sought an improved and inexpensive method for the determination of diclofenac. In the present paper, the search for more available reagents for derivative formation and the assay procedure using those reagents are described.

EXPERIMENTAL

Reagents

All chemicals were of analytical grade, and the organic solvents for extraction were of especially fine grade; these were purchased from Wako Pure Chemicals, Osaka, Japan.

Extraction of diclofenac from plasma

Extraction was carried out with slight modifications of the method previously reported by Geiger et al. [1]. A 0.5-ml volume of plasma was acidified with 1 ml of 2.7 M phosphoric acid, and diclofenac in the solution was extracted with 5 ml of benzene under continuous shaking for 30 min. The mixture was centrifuged at 1000 g for 10 min to separate the phases. A 4-ml aliquot of the benzene extract thus obtained was added to 2 ml of 0.1 N sodium hydroxide solution, and the mixture was stirred with a Vortex mixer. After centrifugation at 1000 g for 10 min, as much of the benzene phase as possible was removed by aspiration and the residual benzene was evaporated under a stream of nitrogen gas. The aqueous phase was acidified with 0.2 ml of 43% phosphoric acid, and diclofenac in the solution was re-extracted with 3 ml of benzene using a Vortex mixer. A 2.5-ml aliquot of the benzene layer in a test-tube was evaporated to dryness at 40°C under reduced pressure. The dried residue was dissolved in diethyl ether, and transferred to a 5-ml ready-made ampoule. The test-tube was washed twice with ether. The ether washes were combined in the ampoule and evaporated to dryness in a gentle stream of nitrogen gas. Diclofenac in the residue was subjected to cyclization or esterification as described below.

Cyclization of diclofenac

The procedure described by Geiger et al. [1] was modified as follows. The dried residue in the ampoule was dissolved in 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid, and the ampoule was sealed. After reaction in a water-bath at 75°C for 75 min, the ampoule was opened, and 0.4 ml of 25% potassium hydrogen carbonate solution and 2 ml of *n*-hexane were added. The mixture was shaken on a Vortex mixer, and 1.5 ml of the hexane phase were transferred to a 2.5-ml test-tube. Then hexane was evaporated to dryness in a gentle stream of nitrogen gas below 40°C. The residue was dissolved in an aliquot of benzene containing aldrin (25 ppb), of which a 1- μ l aliquot was injected into the gas chromatograph.

Esterification of diclofenac

The dried residue in the ampoule was dissolved in 0.15 ml of methanol or

ethanol containing 0.1% (v/v) or 0.5% (v/v) sulfuric acid, respectively, and the ampoule was sealed. After reaction in a water-bath at 60°C for 1 h, the ampoule was opened and treated as described above.

Gas-liquid chromatography

The gas-liquid chromatographic (GLC) analysis was carried out on a Shimadzu Model GC-4CMPFE gas chromatograph, which was operated at a pulse-rate of 10 kHz and a temperature of 290°C. The glass column, 2 m × 3 mm I.D., was packed with 1.5% Silicone OV-17 on Shimalite W AW DMCS, 80–100 mesh. The carrier gas was nitrogen at a flow-rate of 50 ml/min. The electrometer setting was continuously kept at range 10², attenuation 8. The injection port temperature was 290°C, column oven 260°C. The peak height ratio was used for quantitation. Typical gas chromatograms obtained using the present procedures are shown in Fig. 1.

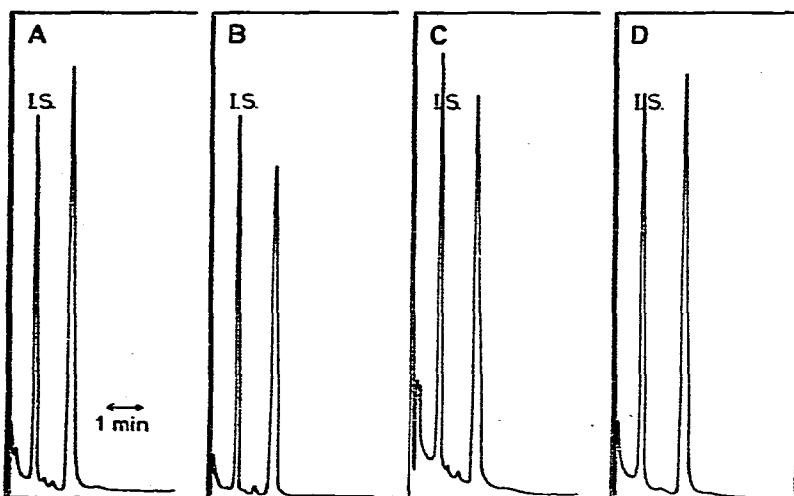


Fig. 1. Typical gas chromatograms, with electron-capture detection, of the reaction products obtained using four different reaction systems: trifluoroethanol–H₂SO₄ (A), trifluoroethanol–methanol–H₂SO₄ (B), methanol–H₂SO₄ (C) and ethanol–H₂SO₄ (D). Analytical conditions were as described in Experimental. I.S., internal standard (aldrin).

RESULTS AND DISCUSSION

Derivative formation from diclofenac

Since we had noticed that the presence of methanol in trifluoroethanol caused about a threefold increase in sensitivity compared to trifluoroethanol alone, the determination of diclofenac in plasma was carried out using the mixture of methanol and trifluoroethanol. Thereafter, we attempted to react diclofenac in methanol or ethanol alone, instead of in trifluoroethanol, in the presence of sulfuric acid under the same reaction conditions of Geiger et al. [1]. Reaction in methanol or trifluoroethanol yielded a single peak with a retention time of 16 min when analyzed by GC-MS under the conditions described above except that the column oven temperature was maintained at 200°C and

the flow-rate of carrier gas (helium) was 40 ml/min; reaction in ethanol yielded a peak at the retention time of 19 min. At this point, it was thought that the indolone derivative and the methyl ester of diclofenac might overlap each other on the gas chromatogram. To analyze the reaction products in five independent reactions, thin-layer chromatography was carried out (Fig. 2). It was shown

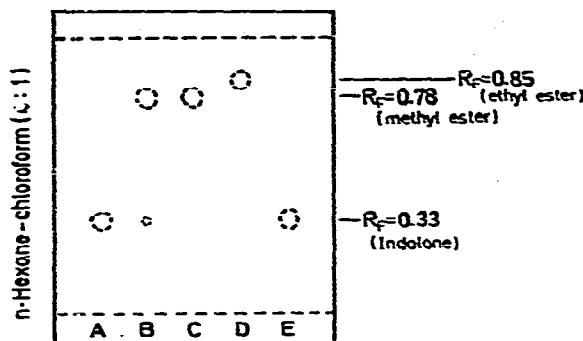


Fig. 2. Thin-layer chromatogram of reaction products obtained in five different reaction systems. Chromatography was performed on silica gel (Wakogel B5FM for UV detection), and each spot was visualized by UV absorption. Diclofenac (1 mg) was reacted with each reagent [trifluoroethanol-H₂SO₄, (A), trifluoroethanol-methanol-H₂SO₄, (B), methanol-H₂SO₄, (C), ethanol-H₂SO₄, (D) or methanol-HCl (E)] and each reaction product extracted with *n*-hexane was subjected to thin-layer chromatography.

that the chief reaction product in a mixture of trifluoroethanol (0.15 ml) and methanol (0.05 ml) is the methyl ester of diclofenac. The reaction products produced in those systems were subjected to GC-MS, and were confirmed to contain the indolone or the ester (see Fig. 3). Geiger et al. tried using diazomethane for the esterification of diclofenac and found the simultaneous formation of the ester and the indolone. We examined the methylation reaction using diazomethane; we detected no indolone, but dimethylated diclofenac in addition to the monoester, which were detected by GC-MS analysis.

Reaction conditions for derivative formation

To establish optimum reaction conditions, the reaction temperature and sulfuric acid concentration were changed in three reaction systems. From the results (Fig. 4), it was concluded that the most preferable conditions were the methanol system with 0.1% (v/v) sulfuric acid, and with reaction temperature and time of 60°C and 1 h, respectively.

Standard curves and calibration curves

Fig. 5 shows standard curves (without extraction procedure) of diclofenac in four systems. The curves are linear up to at least 10 µg. Fig. 6 shows calibration curves for each analytical procedure with the extraction procedure. A good linearity is seen up to at least 10 µg/ml. As little as 0.1 µg of diclofenac can be measured by the present method.

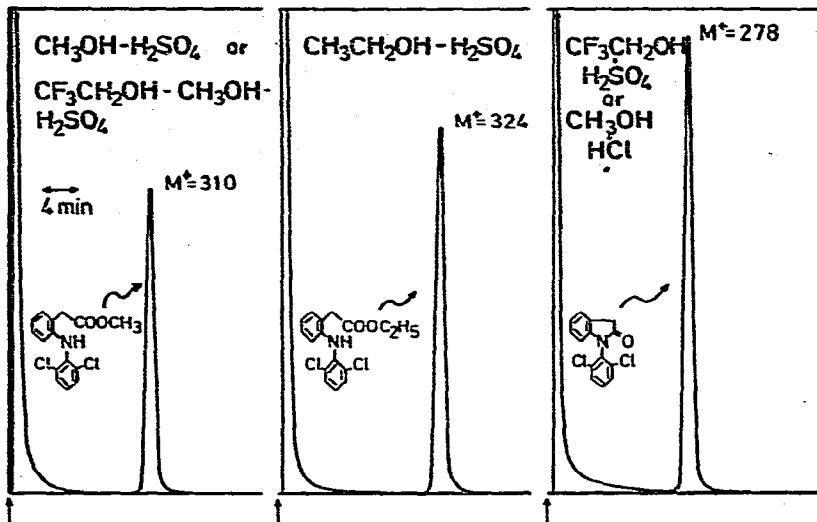


Fig. 3. Gas chromatograms of the reaction products obtained in five different reaction systems by GC-MS. The reaction products obtained as described in Fig. 2 were also subjected to GC-MS, which was performed on a Shimadzu LKB 9000 gas chromatograph-mass spectrometer. The column oven temperature was maintained at 200°C, and the flow-rate of carrier gas (helium) was 40 ml/min. Column packings and size were as described in Experimental. Reaction of diclofenac with methanol- H_2SO_4 or trifluoroethanol-methanol- H_2SO_4 , or methanol-HCl gave the peak at 310 as M^+ (the methyl ester), while reaction with trifluoroethanol- H_2SO_4 or methanol-HCl gave the peak at 278 as M^+ (indolone). Reaction with ethanol- H_2SO_4 gave the peak at 324 as M^+ (the ethyl ester).

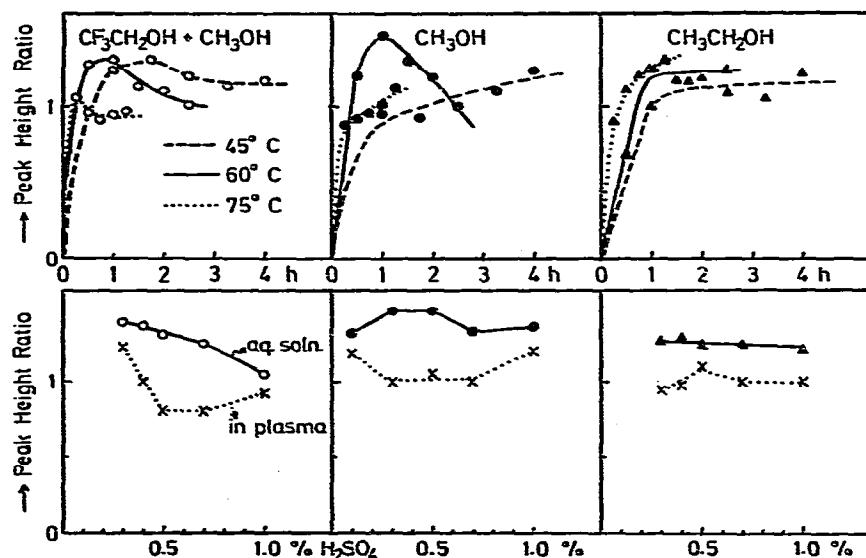


Fig. 4. The effect of temperature and time and sulfuric acid concentration on the esterification of diclofenac. Diclofenac was reacted with 0.15 ml of alcohol containing 0.5% sulfuric acid at three different temperatures (upper panels). To test the optimum concentration of sulfuric acid, diclofenac was reacted with 0.15 ml of alcohol containing various concentrations of sulfuric acid at 60°C for 1 h (lower panels). Extraction and determination were carried out as described in Experimental.

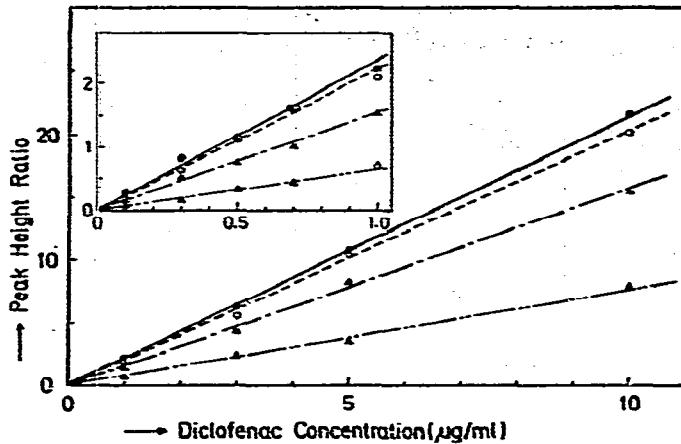


Fig. 5. Standard curves of diclofenac. Varying amounts of diclofenac methanol solution in an ampoule were evaporated to dryness, reacted in four different systems, and measured by GC with electron-capture detection after being dissolved in an aliquot of benzene containing 25 ppb of aldrin. ●—● represents the standard curve obtained by reaction of diclofenac with 0.15 ml of methanol containing 0.1% (v/v) sulfuric acid at 60°C for 1 h, ○—○ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid and 0.05 ml of methanol at 60°C for 1 h, ▲—▲ by reaction with 0.15 ml of ethanol containing 0.5% (v/v) sulfuric acid at 60°C for 1 h, and △—△ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid at 75°C for 75 min.

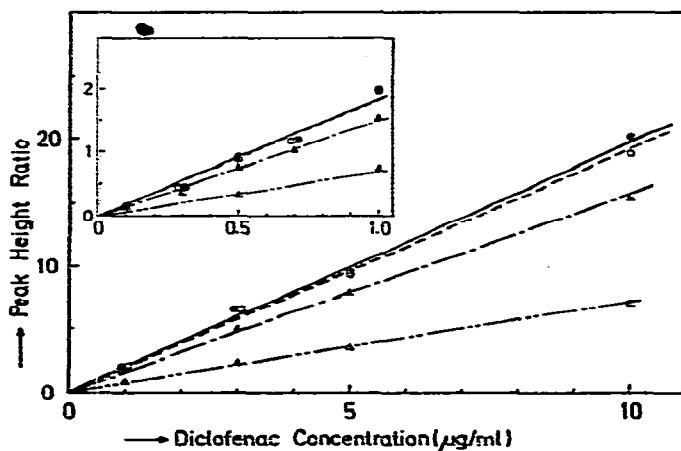


Fig. 6. Calibration curves of diclofenac. Varying amounts of aqueous diclofenac solution were treated as described in Extraction of diclofenac from plasma, then reacted in four different systems and measured by GC with electron-capture detection after being dissolved in an aliquot of benzene containing 25 ppb of aldrin. ●—● represents the calibration curve obtained by reaction with 0.15 ml of methanol containing 0.1% (v/v) sulfuric acid at 60°C for 1 h, ○—○ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid and 0.05 ml of methanol at 60°C for 1 h, ▲—▲ by reaction with 0.15 ml of ethanol containing 0.5% (v/v) sulfuric acid at 60°C for 1 h, and △—△ by reaction with 0.15 ml of trifluoroethanol containing 0.5% (v/v) sulfuric acid at 75°C for 75 min.

Recovery tests

In order to apply the esterification methods to the assay of diclofenac in plasma, recovery tests were carried out. Various amounts of diclofenac were added to each sample of plasma and the amount of diclofenac was measured by the procedure given above. The results are summarized in Table I. Recovery data obtained from the esterification method are comparable with those from the method of Geiger et al. [1].

TABLE I

RECOVERY OF DICLOFENAC ADDED TO PLASMA

Diclofenac extracted from plasma was reacted in three different systems, trifluoroethanol—methanol—H₂SO₄ (B), methanol—H₂SO₄ (C) or ethanol—H₂SO₄ (D). Each value is the mean of duplicate determinations.

Diclofenac added ($\mu\text{g/ml}$)	Diclofenac determined ($\mu\text{g/ml}$)		
	B	C	D
1.0	0.97	0.96	1.00
2.0	1.87	1.84	1.96
4.0	3.64	3.58	3.26
6.0	5.72	6.23	5.79
Recovery (%)	94.1 \pm 2.6	95.2 \pm 6.3	94.0 \pm 8.5

ACKNOWLEDGEMENT

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