Reprinted from
Journal of Chromatography, 168 (1979) 266–272

CHROM. 11,318

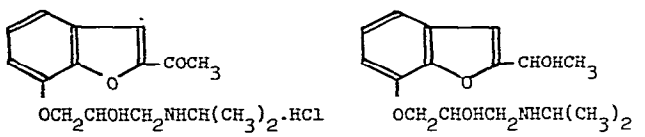
Note

Simultaneous determination of befunolol, a β-blocking agent, and its metabolite in human plasma by gas chromatography with electron-caputre detection

KENJI KAWAHARA and TATSUYA OFUJI
Research Laboratory, Kakenyaku-Kako Co., Ltd., 14 Minami-kawaharamachi, Shinomiya, Yamashina-ku, Kyoto 607 (Japan)
(Received June 27th, 1978)

The development of halogenation methods such as trifluoroacetylation and heptafluorobutyrylation has enabled ultramicro amounts of non-halogenated compounds to be determined by gas chromatography with electron-capture detection (ECD-GC). Very low plasma levels of several β-blocking agents have also been determined by such methods[1–6].

Befunolol hydrochloride (BFE-60), 2-acetyl-7-(2-hydroxy-3-isopropylaminopropoxy)benzofuran hydrochloride, is a β-blocking agent[7] and has the chemical structure as shown in Fig. 1. 2-Hydroxyethyl-7-(2-hydroxy-3-isopropylaminopropoxy)-benzofuran (MI), the reduction product of BFE-60 (see Fig. 1), has been identified as one of the metabolites of BFE-60 excreted in animal and human urine[8], and still has a slightly weaker β-blocking activity than BFE-60[9]. The amount of MI excreted in human urine after oral administration of BFE-60 accounted for 47–76% of dose[8].



BFE-60 MI

Fig. 1. Structures of BFE-60 and MI.

In the course of pharmacodynamic and bioavailability investigations of this drug, it became necessary to develop a highly sensitive and specific method for the determination of plasma levels of BFE-60 and MI. In this work we established an ECD-GC method that is capable of the simultaneous determination of as low as nanogram plasma levels of both compounds.

EXPERIMENTAL

Reagents and materials

Benzene, ethyl acetate, diethyl ether, *n*-hexane (for pesticide residue analysis),

ethylene dichloride (for spectroscopy) and trifluoroacetic anhydride (TFA, for biochemical research) were obtained from Nakarai Chemicals (Kyoto, Japan). 2-Acetyl-7-(2-hydroxy-3-*sec*.-butylaminopropoxy)benzofuran hydrochloride, used as an internal standard (IS), was synthesized in this laboratory. Aldrin, used as an external standard (ES), was obtained from Wako (Osaka, Japan). The IS and ES were used as aqueous (50 ng/ml) and benzene (40 ng/ml) solutions, respectively.

GC conditions

A Shimadzu 4BM gas chromatograph equipped with a 10-mCi nickel-63 electron capture detector was used. A U-shaped glass column (1 m × 3 mm I.D.) was packed with 3% OV-25 on Chromosorb W AW DMCS (80–100 mesh). The column temperature was 210°, the injection port and detector temperature 285° and the carrier gas (nitrogen) flow-rate 30 ml/min.

Sample preparation

Extraction from aqueous solution and plasma. In a glass-stoppered test-tube are placed 0.1–2.0 ml of sample (aqueous solution or plasma), 2.0 ml of IS solution and 0.5 ml of 3 *N* sodium hydroxide solution. The mixture is extracted twice with 5 ml of diethyl ether by shaking for 30 sec and centrifuging for 5 min at 1660 *g*. The combined ether layers are transferred to another test-tube and back-extracted with 5 ml of 0.5 *N* hydrochloric acid by shaking for 30 sec. After centrifugation for 2.5 min at 1660 *g*, the ether layer is removed by aspiration. The remaining acidic aqueous layer is washed three times with 5-ml portions of diethyl ether, made alkaline with 1.2 ml of 3 *N* sodium hydroxide solution, extracted twice with 5 ml of diethyl ether by shaking for 30 sec, and centrifuged for 2.5 min at 1660 *g*. The combined ether layers are evaporated to dryness in a flask under reduced pressure at 40°. The residue is submitted to trifluoroacetylation followed by GC analysis.

Trifluoroacetylation. The dried residue containing 100 ng of BFE-60, MI and IS is dissolved in 50 μl of ethyl acetate and 150 μl of TFA. After standing for 1 min at room temperature, 150 μl of benzene and 10 ml of ice-cold 0.01 *N* hydrochloric acid are added and the reaction mixture is shaken for 5 sec. A 200-μl volume of ES solution is added to the mixture, which is shaken for 5 sec. The mixture is transferred into a 10-ml volumetric flask and a 2-μl portion of the organic layer that separated on the neck of the flask is injected into the gas chromatograph.

Drug administration to volunteers

Two healthy male adults, 23 and 42 years of age, weighing 51 and 59 kg, each received 20 mg BFE-60 as a capsule after a 16 h fast. One week after the administration, the same volunteers again received the same dose of BFE-60 30 min after breakfast. A 7-ml volume of blood was withdrawn using a heparinized syringe at 0, 1, 2, 3, 4 and 6 h after administration of the drug, and 0.5–2 ml of plasma was used for analysis.

RESULTS AND DISCUSSION

The extractabilities of BFE-60 and MI were examined using diethyl ether, ethylene dichloride and *n*-hexane as extraction solvents. An aqueous solution containing of 20 μg/ml of BFE-60 or MI was adjusted to pH 1, 8 and 13 using 0.2 *N*

hydrochloric acid, 0.2 *M* sodium hydrogen carbonate solution and 0.2 *N* sodium hydroxide solution. To 10 ml of each of these solutions the same volume of organic solvent was added and the mixtures were shaken for 10 min at room temperature. The absorbances of the organic and aqueous layers were measured at 295 nm for BFE-60 and 245 nm for MI.

The results (Table I) indicate that the extraction ratios of BFE-60 and MI are dependent on the pH of the aqueous layer and the polarity of the organic solvent. Diethyl ether and ethylene dichloride showed higher extractabilities from basic solution than *n*-hexane. The extraction ratio of MI with diethyl ether was almost equal to that of BFE-60 with diethyl ether, and diethyl ether was therefore used as the extraction solvent in this study. BFE-60 and MI in acidic aqueous solution (pH 1) were not extracted into diethyl ether. Therefore, a single procedure was adequate for back-extraction of BFE-60 and MI into the aqueous layer from the organic layer.

TABLE I

SELECTION OF ORGANIC SOLVENT FOR EXTRACTION OF BFE-60 AND MI FROM AQUEOUS SOLUTION

The extraction ratio was calculated from the formula

$$\frac{\text{Absorbance of organic layer}}{\text{Absorbance of aqueous layer} + \text{Absorbance of organic layer}} \cdot 100$$

Organic solvent	*pH of aqueous layer*	*Extraction ratio (%)*	
		BFE-60	*MI*
Diethyl ether	13	99	89
	8	58	53
	1	0	0
Ethylene dichloride	13	100	85
	8	93	48
	1	0	0
n-Hexane	13	20	2
	8	0	0
	1	0	0

The trifluoroacetylated products of authentic BFE-60, MI and the IS gave single peaks that were completely separated on OV-1, OV-17 and OV-25 columns. However, a minor interfering peak was observed on chromatograms of the plasma extracts when separated on OV-1 and OV-17. Fig. 2 shows the chromatograms of the plasma extracts separated on an OV-25 column. The retention times of BFE-60, MI and the IS were 3.4, 6.8 and 8.5 min, respectively.

Two different procedures, extraction (see Experimental) and evaporation, were examined for removing excess amounts of TFA from the reaction mixture because the ECD is highly sensitive to this reagent. The evaporation of TFA was achieved by heating the reaction mixture at 40° under reduced pressure. Table II shows the peak-height ratios of BFE-60 and MI to the IS and ES. The ES was used as a secondary standard to investigate the effect of the reaction condition on the yield of trifluoroacetyl derivatives of BFE-60, MI and the IS, because the ES does not react with TFA

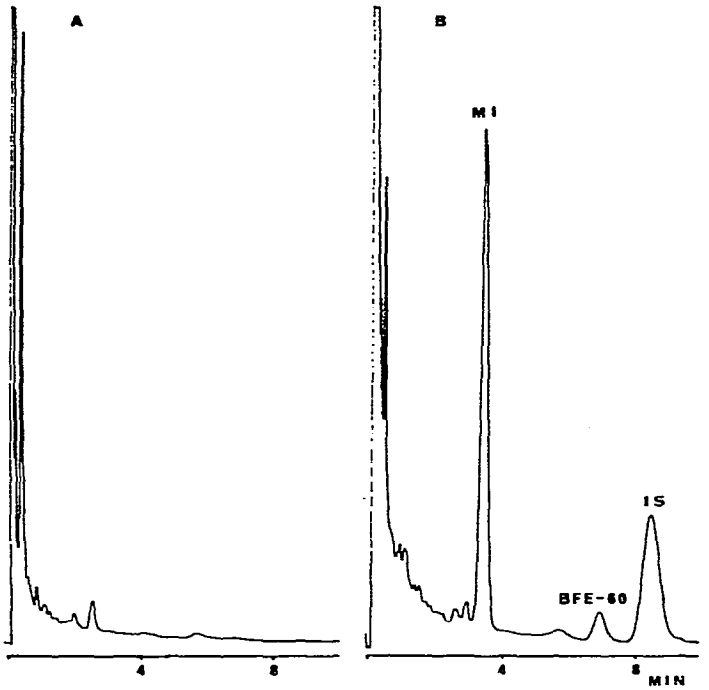


Fig. 2. Chromatograms of human plasma extracts. A, Normal plasma; B, plasma from a volunteer who received a single oral dose of 20 mg BFE-60.

TABLE II

PEAK-HEIGHT RATIO AND ITS REPRODUCIBILITY IN DIFFERENT METHODS OF REMOVAL OF EXCESS OF REACTION REAGENT

Each value represents the mean and standard error of 5 experiments.

Removal method		*BFE-60*	*MI*	*IS*
Evaporation	Ratio to ES	0.55 ± 0.02	0.93 ± 0.13	0.32 ± 0.01
	Ratio to IS	1.74 ± 0.03	0.90 ± 0.37	
Extraction	Ratio to ES	0.58 ± 0.01	1.45 ± 0.03	0.35 ± 0.01
	Ratio to IS	1.67 ± 0.01	4.12 ± 0.07	

and is not affected by removal of excess of reagent. Therefore, peak-height ratios of BFE-60, MI and the IS to the ES were used as an indication of the reproducibility of the method. It can be seen from Table II that the two methods give almost same values for the peak-height ratios of BFE-60 and the IS to the ES, whereas the peak-height ratio of MI to the ES in the extraction method is high with a small variation in comparison with that obtained by the evaporation method. This suggests that trifluoroacetylated MI may be partly decomposed or vaporized during evaporation under reduced pressure. Thus, the extraction method is preferable for the simultaneous determination of BFE-60 and MI. Moreover, the extraction procedure requires a

shorter time than the evaporation procedure, the former being suitable for the analysis of a large number of specimens.

The time and temperature dependencies of trifluoroacetylation were investigated using 1 ml of aqueous solution containing 100 ng each of BFE-60, MI and the IS. The residue obtained from the solution was dissolved in 50 μl of ethyl acetate and 150 μl of TFA. After reaction for 1, 5 and 10 min at 15° and 35°, the excess amounts of TFA were removed by the extraction method. Fig. 3 shows peak-height ratios of BFE-60, MI and the IS to the ES, indicating that reaction for 1 min at room temperature is sufficient for trifluoroacetylation.

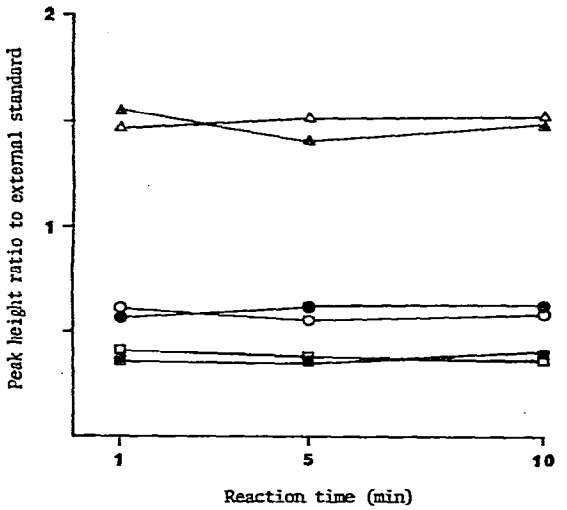


Fig. 3. Effects of reaction time and temperature on trifluoroacetylation of BFE-60, MI and the IS. 15°: △, MI; ○, BFE-60; □, IS. 35°: ▲, MI; ●, BFE-60; ■, IS.

A 1-ml aliquot of an aqueous solution containing 5–100 ng/ml of BFE-60 and MI was treated as described under *Extraction from aqueous solution and plasma*. The residue was dissolved in 50 μl of ethyl acetate and treated with 150 μl of TFA. After reaction for 1 min at room temperature, 150 μl of benzene and 10 ml of ice-cold 0.01 *N* hydrochloric acid were added to the reaction mixture, which was shaken for 5 sec and transferred into a 10-ml volumetric flask. A 1–2-μl portion of the organic layer that separated on the neck of the flask was injected into the gas chromatograph. The calibration graphs of BFE-60 and MI on the basis of peak-height ratio were linear, as shown in Fig. 4 (BFE-60, $r = 0.998$; MI, $r = 0.988$). The minimum detectable concentrations of BFE-60 and MI were 2.5 ng/ml using 2 ml of sample. A 2-ml sample of plasma containing 2.5–50 ng/ml of BFE-60 and MI was treated in a similar manner to aqueous solution. The overall recoveries of BFE-60 and MI, as shown in Table III, were 100.9 $\pm$ 2.7% and 102.2 $\pm$ 3.0%, respectively. A high recovery with satisfactory precision was obtained for all the plasma levels of BFE-60 and MI tested. These results suggest that 2-acetyl-7-(2-hydroxy-3-*sec*.-butylaminopropoxy)benzofuran hydrochloride is a suitable internal standard.

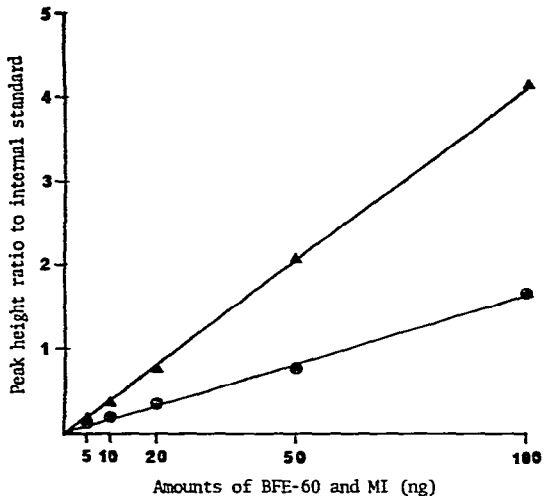


Fig. 4. Calibration graphs for BFE-60 (●) and MI (▲).

TABLE III

RECOVERY OF BFE-60 AND MI FROM PLASMA

Each value represents the mean and standard error of 4 experiments.

Amount added to plasma (ng/ml)		*Amount found (ng/ml)*	
BFE-60	*MI*	*BFE-60*	*MI*
5.0	5.0	5.7 ± 0.6	5.8 ± 0.3
10.0	10.0	10.2 ± 0.6	10.1 ± 0.4
20.1	20.0	19.8 ± 0.8	19.6 ± 1.8
50.2	50.1	46.7 ± 1.1	49.6 ± 4.6
100.4	100.1	101.5 ± 3.6	98.6 ± 2.2

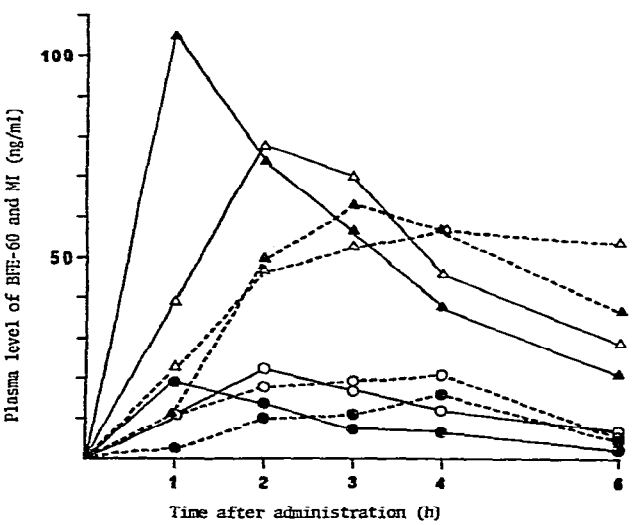


Fig. 5. Plasma levels of BFE-60 and MI after a single oral dose of 20 mg of BFE-60 to human volunteers. Solid lines, fasting state; broken lines, non-fasting state. Volunteer A: ○, BFE-60; △, MI. Volunteer B: ●, BFE-60; ▲, MI.

Fig. 5 illustrates the time courses of plasma levels of BFE-60 and MI in two volunteers after a single oral administration of 20 mg of BFE-60. The plasma levels of both BFE-60 and MI reached maxima 1–2 h after administration in the fasting state and 3–4 h in the non-fasting state. The difference in the time to the absorption maximum indicates the gastric emptying effect.

The plasma levels of MI were 2–12 times higher than those of BFE-60. The biological half-lives of BFE-60 in volunteers A and B in a fasting state were 1.62 and 2.25 h, respectively.

CONCLUSION

The method described is highly sensitive, specific and reproducible, and permits the simultaneous determination of nanogram levels of BFE-60 and MI in human plasma.

ACKNOWLEDGEMENTS

The authors thank Prof. T. Uno and Dr. T. Nakagawa (Faculty of Pharmaceutical Sciences, Kyoto University) for many suggestions during this work.

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