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COMPARISON OF GAS CHROMATOGRAPHIC—ELECTRON-CAPTURE DETECTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF BUTOFILOLOL IN BIOLOGICAL FLUIDS

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SUMMARY

Two selective and sensitive methods for the quantitative analysis of butofilol in human plasma and urine are discussed. The first method is a gas chromatographic assay with electron-capture detection using extraction with toluene, several clean-up procedures and derivatization. The second method is based on high-performance liquid chromatography and a single extraction with dichloromethane. The two assay methods were applied to the determination of the same human plasma samples after administration of a single, oral 200-mg dose of butofilolol. A good correlation between the results (inter-laboratory comparison) is obtained, validating both techniques.

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INTRODUCTION

Butofilolol (CAFIDE®), a new β-adrenoceptor blocking agent (Fig. 1), was found to be highly effective for the treatment of hypertension [1, 2]. Animal pharmacokinetic studies were performed in the baboon using intravenous and oral administration of the ^{14}C-labelled drug. Absorption was complete but, due to an extensive first-pass hepatic metabolism, relatively low plasma levels of the parent drug were observed after oral administration [3]. Therefore human pharmacokinetic investigations required a very sensitive and selective analytical procedure for the quantitative determination of the parent drug in plasma and urine.

First, a gas chromatographic (GC) assay with electron-capture detection (ECD) of the diheptafluorobutyrate derivative of butofilolol was developed. However, this method required several clean-up procedures and was time consuming. A more simple and rapid method was developed, using a single-step extraction with high-performance liquid chromatographic (HPLC) separation.

The GC–ECD and HPLC methods are described below. The precision and reproducibility of each procedure were evaluated. The two methods were applied to the analysis of butofilolol in identical plasma samples collected from a volunteer after a single, 200-mg oral administration of the drug. Comparison of the results allowed evaluation of the respective advantages and disadvantages of the two methods.

EXPERIMENTAL

Reagents

Organic solvents used were toluene, dichloromethane and methanol. All solvents were analytical grade and were obtained from Merck (Darmstadt, F.R.G.). Toluene was glass-distilled prior to use. The other solvents were used without prior distillation.

Heptafluorobutyric anhydride (Merck) was used for derivatization before GC—ECD analysis. The reagent was freshly purified by distillation over excess phosphorus pentoxide for 3 h, then kept under anhydrous conditions until use. Trimethylamine solution (1.2 *M* in toluene) was freshly prepared before each experiment from recrystallized trimethylammonium chloride (Merck).

The inorganic reagents were all prepared in distilled water. Sodium hydroxide, sodium bicarbonate and dipotassium hydrogen phosphate were obtained from Merck.

Standard solutions

Butofilolol as maleate (CM 6805 a) and its internal standard (CM 6859) (Fig. 1) were obtained from Sanofi Research/Center of Montpellier (France). Standard solutions of these compounds were freshly prepared each day at suitable dilution in phosphate buffer, pH 7.4.

Glassware

All glassware used in the extraction and derivatization procedures was washed with sulphochromic acid, rinsed with deionized water and finally dried

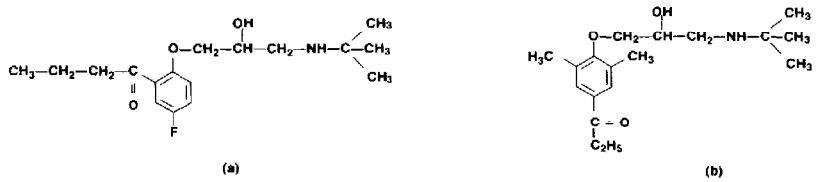

Fig. 1. Molecular structures of: (a) butofilolol and (b) its internal standard.

at 60°C. For the GC–ECD procedure, glassware was silanized with 2% dimethyldichlorosilane (Fluka, Paris, France) in toluene, rinsed with toluene and then methanol and dried at 60°C.

Sample preparation

GC–ECD procedure. A 1-ml sample of plasma or urine was added to a glass-stoppered 10-ml centrifuge tube containing 1 ml of an aqueous solution of the internal standard (500 ng) and 0.2 ml of 1.0 *M* carbonate buffer, pH 12 (1 *M* sodium carbonate–0.5 *M* sodium hydroxide). After shaking with 6 ml of toluene for 15 min and centrifuging at 2500 *g* for 5 min, 5 ml of the organic phase were transferred to another tube containing 4 ml of 0.1 *M* phosphate buffer, pH 2.0. This tube was shaken and centrifuged under the same conditions. Toluene was discarded and a 3-ml aliquot of the acidic phase was transferred to another tube, made alkaline (pH 12) with 0.2 ml of carbonate buffer (1 *M* Na_2CO_3–5 *M* sodium hydroxide) and re-extracted with 6 ml of toluene. After centrifugation, 5 ml of the toluene phase were transferred into a small glass tube and evaporated to dryness under a stream of nitrogen.

The dry residue was reconstituted in 1 ml of toluene and 0.1 ml of 1.5 *M* trimethylamine, then derivatized with 50 μl of heptafluorobutyric anhydride for 5 min in an ice-bath. Then excess of reagents was removed by clean-up with 3 ml of 0.5 *M* phosphate buffer, pH 6.0. The phases were separated by centrifugation, and 1–5 μl of the toluene phase were injected.

HPLC procedure. A 1-ml sample of plasma or urine was spiked with internal standard and made alkaline under the same conditions as described for the GC–ECD procedure in the first extraction step. Dichloromethane was used as organic solvent instead of toluene. Tubes were vortexed for 1 min and centrifuged at 2500 *g* for 15 min. A 5-ml aliquot of the organic layer was transferred to another tube. Extraction of the sample was repeated a second time under the same conditions, and a second 5-ml aliquot of the dichloromethane phase was separated. Then, the combined organic phase was evaporated to dryness under a stream of dry nitrogen at room temperature. The residue was dissolved in 50 μl of the mobile phase and injected.

Apparatus and chromatographic conditions

For GC–ECD analysis, a Hewlett-Packard 5710 A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The glass column (180 × 0.4 cm I.D.) was filled with 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh). The chromatograph was operated isothermally with the oven, detector and injection-port temperatures maintained at 230, 300 and 250°C,

respectively. The carrier gas was 10% methane in argon at a flow-rate of 30 ml/min. The chromatograph was connected to a recorder with a scale range of 1 mV (Sefram type 1.10 PE; Paris, France).

The mass spectrometric analysis of the butofilolol derivative was performed using a gas–liquid chromatograph coupled on-line to a mass spectrometer (Ribermag 10-10 B) operating in the chemical-ionization mode with ammonia as reagent gas.

HPLC analyses were performed using a 6000A pump, a U6K sample injector, a UV filter (313 nm) spectrometer M 440 equipped with an 8-μl capacity flow cell (all from Waters). A 250-mm steel column was used, packed with a monomolecular layer of octadecyltrichlorosilane, chemically bonded to Porasil beads with an average particle size of 10 μm (Bondapak C_{18}, Waters). The mobile phase was a mixture of methanol–water–Pic B 7 (Waters) (60:40:1) with a flow-rate of 2 ml/min.

All materials used throughout the analyses, especially those used for the GC–ECD procedure, were made of glass to avoid interaction between biological material and plasticizers [4].

Biological sampling

Blood (about 7 ml) from one patient receiving a single 200-mg oral dose of butofilolol was collected on heparinized glass centrifuge tubes. The plasma samples were immediately separated by centrifugation, then stored at −20°C until analyzed.

Calibration and quantitation

Quantitation of butofilolol was achieved on the basis of a calibration curve. Standard curves were run daily by spiking 1.0 ml of blank plasma with a known amount of internal standard and increasing amounts of butofilolol. A least-squares regression between concentration and peak-height ratios of the drug to the internal standard was calculated.

RESULTS AND DISCUSSION

GC–ECD method

The use of toluene as extraction solvent at pH 12 provided less interfering substances in the chromatogram in comparison to heptane, benzene or ethyl ether.

Due to the probable steric hindrance of the *tert.*-butyl radical on the nitrogen atom and the acyl radical in the *ortho*-position of the side chain of butofilolol, the derivatization step was critical and needed the use of a catalyst to improve the yield of the reaction. The acylation of butofilolol and its internal standard with heptafluorobutyric (HFB) anhydride provided derivatives with excellent properties for GC analysis and high response to electron-capture detection.

The reaction was improved using trimethylamine (TMA) in toluene at 0°C in an ice-bath. Under these conditions, the derivatization was optimal after 5 min. The structures of the HFB derivatives were confirmed by mass spectrometry with chemical ionization (Fig. 2). The molecular ion of the

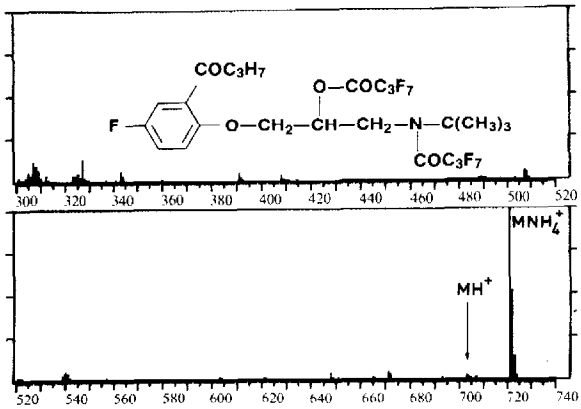

Fig. 2. Mass spectrum of the HFB derivative of butofilolol.

parent drug derivative observed at *m/e* 721 was consistent with the derivatization product proposed.

Attention should be paid during extraction and derivatization steps when handling solutions of the drug in its basic form in solvents of low polarity such as toluene. Indeed, adsorption on glass walls was observed, and the use of pre-treated glassware was necessary. Consequently, all glassware was silanized to avoid irreproducible losses by adsorption.

The overall recovery (extraction from plasma and derivatization) was estimated to be 85—90%. This yield was constant over the concentration range to be considered (10—2000 ng/ml). Usually, the calibration graphs were made in the concentration range 25—500 ng/ml with 500 ng of internal standard. A least-squares regression analysis of the fit between the peak-height ratios of the sample substance and the internal standard versus amounts of substance added was applied. An example of such a regression line was: $y = 0.03349x - 0.02203$, with a correlation coefficient, r, of 0.9999, where y corresponds to the peak-height ratio and x to the drug concentration. The calibration curves were linear within the range used. The limit of detection of butofilolol was estimated to be 20 ng/ml.

Examples of chromatograms obtained after analysis of blank plasma and spiked plasma are illustrated in Fig. 3. Under the chromatographic conditions used, the two peaks corresponding to butofilolol and internal standard derivatives were well resolved. No interfering peaks appeared in the analysis of plasma samples from several patients.

The results of the reproducibility are reported in Table I. The coefficient of variation ranged from 13.1 to 1.5%. The average reproducibility of the assay over the concentration range studied was 6.3%.

HPLC method

Examples of HPLC chromatograms are shown in Fig. 4. No interfering peaks due to endogenous compounds were observed.

A typical standard curve obtained responded to the following equation: $y = (0.0047 \pm 0.00001)x + (0.15 \pm 0.10)$, where y corresponds to the peak-height ratio and x to the drug concentration, with a correlation coefficient, r,

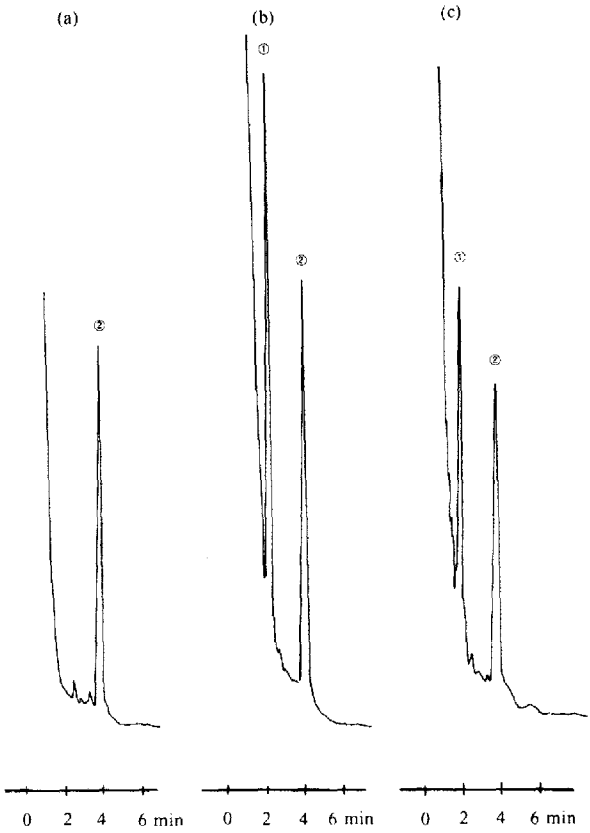

Fig. 3. GC—ECD chromatograms from human plasma samples: (a) blank control; (b) spiked with 250 ng of butofilolol and 250 ng of internal standard; and (c) plasma obtained from a patient receiving a 100-mg oral dose of butofilolol. 1 = Internal standard; 2 = butofilolol.

TABLE I

PRECISION AND ACCURACY OF THE ANALYSIS OF BUTOFILOLOL USING THE GAS CHROMATOGRAPHIC—ELECTRON-CAPTURE METHOD

Theoretical concentration of butofilolol (ng/ml)	Measured concentration of butofilolol (ng/ml)*	Coefficient of variation (%)
50	54.2 ± 7.1	13.1
100	98.8 ± 5.9	5.9
200	190 ± 9.3	4.9
500	463 ± 7.0	1.5

*Values are the mean ± the standard deviation (n = 7).

of 0.9995. These data indicated that the peak-height ratio at the origin was not significantly different from zero. This confirmed the absence of endogenous interfering peaks and the linearity of the detector response within the concentration range studied.

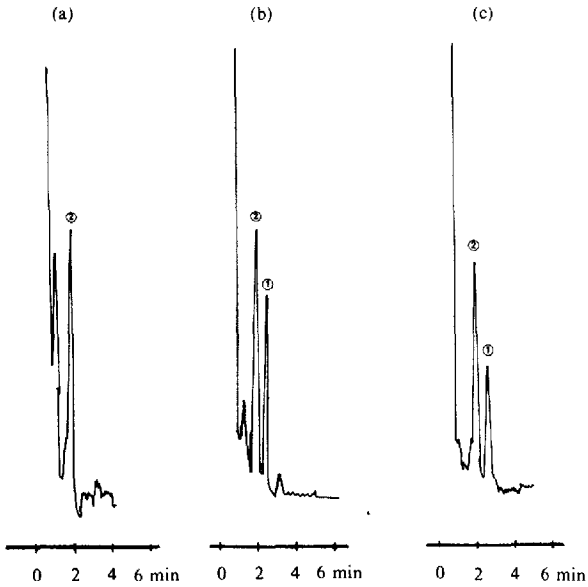

Fig. 4. Typical HPLC chromatograms of human plasma: (a) before administration (control); (b) spiked with 100 ng/ml of butofilolol; (c) plasma obtained from a patient receiving a 200-mg oral dose of butofilolol. 1 = Butofilolol; 2 = internal standard.

Extraction recovery was calculated using the peak-height ratio obtained after direct injection of pure solutions and after extraction. The extraction recovery decreased from 85% for the lowest concentration (20 ng/ml) to 67% for the highest one (2000 ng/ml).

The recovery of the drug from the entire procedure was determined from spiked plasma samples (n = 10) at several concentrations (Table II). Mean results appeared to be very close to the theoretical concentrations, showing suitable recovery and accuracy. The standard deviation was at a minimum of 3.1% for the 2000 ng/ml level. The limit of detection was about 20 ng/ml.

TABLE II

PRECISION AND ACCURACY OF THE ANALYSIS OF BUTOFILOLOL USING THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

Theoretical concentration of butofilolol (ng/ml)	Measured concentration of butofilolol (ng/ml)*	Coefficient of variation (%)
20	23.5 ± 3.4	14.3
50	48.6 ± 6.4	13.2
100	117.3 ± 10.6	9.0
200	191.4 ± 12.2	6.4
500	501.8 ± 43.9	8.7
1000	998.0 ± 46.0	4.6
2000	2000.0 ± 62.0	3.1

*Values are the mean ± the standard deviation (n = 10).

Comparison of GC—ECD and HPLC methods

The applicability of the two analytical methods was assessed and compared using the same set of human plasma samples. Fig. 5 summarizes the results obtained for the samples collected after a single, 200-mg oral administration of butofilolol. A fit for the data from Fig. 5 was obtained by linear regression. The equation obtained was $y = 1.148x - 32.2$, where y = concentration obtained after the GC-ECD analysis and x = concentration obtained after HPLC analysis; the correlation coefficient, r, was 0.991 ($p < 0.001$) ($n = 14$). This good correlation indicated that the two methods gave very similar results.

An example of the time course of the butofilolol concentration in plasma in one subject was shown in Fig. 6. The maximum plasma level of about 350 ng/ml was reached 1.25 h after administration, showing the rapid absorption of the drug from the gastrointestinal tract. The plasma concentration—time

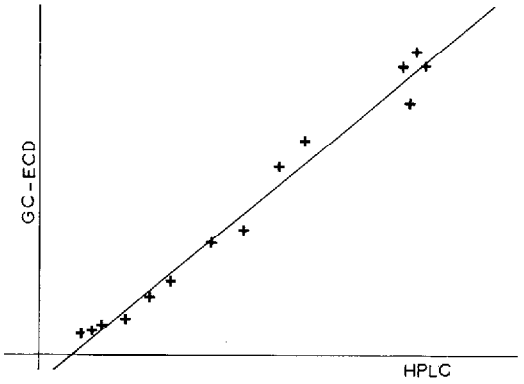

Fig. 5. Least-squares regression line between butofilolol concentrations measured in the same samples by the two different methods.

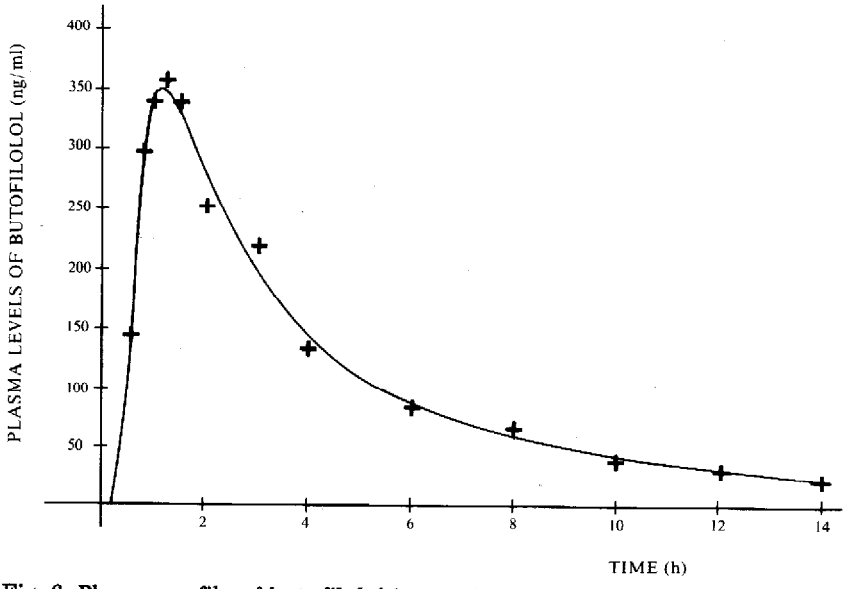

Fig. 6. Plasma profile of butofilolol in a patient following a single, 100-mg oral dose.

curve was adequately fitted to an open two-compartment pharmacokinetic model. The apparent elimination half life of the drug was about 2.4 h. This figure demonstrates that the two methods are sensitive enough for quantitative determination of butofilolol in biological samples. The limit of detection of butofilolol was similar for the two methods (20 ng/ml).

The inter-laboratory comparison of butofilolol plasma levels was very satisfactory considering that two different techniques were used on the same samples. The sensitivity and precision of the two methods were in the same order of magnitude. However, the GC—ECD method requires back extraction, derivatization and several clean-up procedures, and was also time consuming. The HPLC technique appears to be easier for human pharmacokinetic and drug monitoring studies of butofilolol.

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