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DETERMINATION OF BUFURALOL AND ITS MAJOR METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of bufuralol, a benzofuran analogue, in plasma is described.

The unchanged drug, the major metabolites and an internal standard are extracted from plasma, purified by back-extraction steps and thereafter separated using a reversed-phase liquid chromatographic system. The detection is carried out by means of a fluorescence detector and an UV detector connected in series. The sensitivity of the assay for the unchanged drug and the major metabolite is about 1 ng/ml plasma using a 0.5 ml specimen per analysis and the relative standard deviation of the whole assay lies in the range ± 4—5%.

The procedure was successfully used to determine plasma levels in volunteers following a single oral dose of 40 mg of bufuralol. The results obtained using the new high-performance liquid chromatographic method were compared with those determined by another method which combines gas chromatography with mass fragmentography, and it was found that these two sets of results coincided quite well.

INTRODUCTION

The benzofuran derivative bufuralol, 7-ethyl-α[(*tert.*-butylamino)-methyl]-2-benzofuran methanol·HCl (substance I in Table I), a β-adrenergic blocking agent, is under clinical investigation for the treatment of cardiac arrhythmias, angina pectoris and hypertension. Studies in animals and man have shown the drug to be rapidly metabolized [1]. The major metabolites found in human plasma are the carbinol (II), the ketone (III) and the phenol (IV) (Table I). The carbinol and ketone metabolites have been synthesized and show β-blocking activity comparable to the unchanged drug. Therefore, pharmacokinetic studies in man should include the determination of these two metabolites, as well as of the unchanged drug. A spectrofluorimetric assay following thin-layer chromatography for the determination of bufuralol and the carbinol has been published [2]. More recently the determination of bufuralol and its metabolites in plasma by mass fragmentography and by gas chromatography

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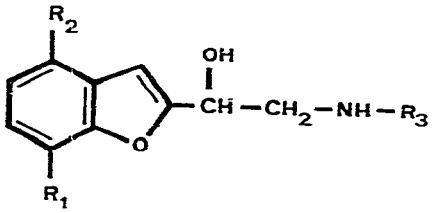
TABLE I

STRUCTURE OF BUFURALOL AND METABOLITES

Substances I—III and the internal standard were used as their racemates.



	R_1	R_2	R_3
Substance I, bufuralol	CH_2-CH_3	H	$C(CH_3)_3$
Substance II, carbinol metabolite	CHOH-CH_3	H	$C(CH_3)_3$
Substance III, ketone metabolite	CO-CH_3	H	$C(CH_3)_3$
Substance IV, phenol metabolite	CH_2-CH_3	OH	$C(CH_3)_3$
Internal standard (I.S.)	CH_2-CH_3	H	$CH(CH_3)_2$

with electron-capture detection has been described [3]. In the last few years, high-performance liquid chromatography (HPLC) has proved to be a successful method in the analysis of drugs and metabolites in biological fluids. Since the published procedures for the determination of bufuralol and its metabolites demand either a highly specialized equipment or are rather complicated to perform, a new assay using HPLC has been developed.

Bufuralol (I) and the carbinol metabolite (II) show fluorescence in solution, which can be used for their detection in HPLC. The ketone metabolite (III) does not fluoresce, but can be quantitated by monitoring its UV absorbance in the eluent. No reference substance was available for the phenol metabolite (Table I); therefore it was not possible to develop the HPLC method for this substance.

EXPERIMENTAL

Reagents

All reagents were of analytical grade purity. The diethyl ether used for extraction was distilled and stored at 4°C. Methanol for preparation of the mobile phase was of spectro grade quality (Uvasol®; Merck, Darmstadt, G.F.R.) and distilled prior to use.

Mobile phase for HPLC: 125 ml of an aqueous* 0.02 *M* $(NH_4)_2HPO_4$ solution, (pH adjusted to 9.2 with ammonia) were mixed with 375 ml of methanol. The mobile phase was degassed immediately before use by pulling a vacuum on the reservoir for a few minutes.

*Bidistilled water has to be used.

Standard solutions

Ten milligrams each of bufuralol, the carbinol metabolite, the ketone metabolite and the internal standard (I.S.; Table I) were weighed into separate amberized 10-ml volumetric flasks and dissolved in methanol to give stock solutions containing 1 mg/ml. Starting from these stock solutions separate dilutions, containing 10 μg/ml of substances I, II, and III, or 2.5 μg/ml of the internal standard, were prepared by diluting aliquots of the corresponding stock solutions with methanol. An intermediate mixture containing 1 μg/ml each of bufuralol (I) and carbinol (II) or 2 μg/ml of ketone (III), was made by pipetting 1 ml, or 2 ml, of the diluted standard solutions into a 10-ml amberized volumetric flask and adjusting to volume with methanol. With this mixture (M) and a 2.5 μg/ml internal standard solution, working standards were prepared as given in Table II.

TABLE II

WORKING STANDARDS

Solution No.	Aliquots of solution (ml)		Diluted with methanol to (ml)	Concentration (ng per 40 μl)			
	I.S.	M		Bufuralol (I)	Carbinol (II)	Ketone (III)	Internal standard
1	0.5	5	10	20	20	40	5
2	0.5	2.5	10	10	10	20	5
3	1	2.5	20	5	5	10	5
4	1	1.25	20	2.5	2.5	5	5
5	1	0.5	20	1	1	2	5

Plasma standards

With the diluted stock solutions in methanol containing 10 μg/ml each of substances I, II and III, an intermediate mixed standard solution with water was prepared by pipetting 4 ml of the bufuralol and the carbinol, or 8 ml of the ketone solution, into a 20-ml volumetric flask and adjusting to volume with water. A 2-ml volume of this solution was added to a 20-ml volumetric flask and diluted to volume with blank plasma. Starting from this plasma stock solution further dilutions according to Table III were prepared. The plasma standards were stored in 1—2 ml portions at —20°C.

Instrumentation

The HPLC system consisted of an Altex (Berkeley, CA, U.S.A.) Model 100 controlled reciprocating pump, a Waters (Milford, MA, U.S.A.) U-6K injector, a Uvikon (Kontron, Zürich, Switzerland) LCD 725 UV detector and a Labotron (Kontron) SFM 22 fluorimeter. The two detectors were connected in series directly after the column, first the UV detector and second the fluorimeter. The column (250 mm × 3.2 mm I.D.) was stainless steel and packed with Spherisorb ODS (Phase Separations, Queensferry, Great Britain) 5 μm particle size.

TABLE III

PLASMA STANDARDS

The plasma standards were prepared and stored in 1—2-ml portions at —20°C.

Plasma standard No.	Concentration (ng/ml plasma)		
	Bufuralol (I)	Carbinol (II)	Ketone (III)
1 (stock solution)	200	200	400
2	100	100	200
3	50	50	100
4	25	25	50
5	10	10	20
6	5	5	10*
7	2.5	2.5	5*

*The detection limit of the ketone metabolite is 10 ng/ml of plasma. These concentrations are near or below this limit.

HPLC operating conditions

The solvent flow-rate used was 0.6 ml/min at a pressure of 150 bar. The UV detector was operated at 231 nm, the cell volume was 8 μl, the path length 6 mm, time constant 1. With the sensitivity set at 1×10^{-2} a.u.f.s., 60 ng of the ketone gave nearly a full-scale pen response on a W + W recorder (Kontron) Model 1200 (dual-channel recorder) set at 5 mV. The fluorimeter flow-cell had a volume of 25 μl; the excitation wavelength was 250 nm, the emission wavelength 300 nm. The instrument was operated at high sensitivity. Twenty nanograms of bufuralol, 10 ng of the carbinol metabolite and 20 ng of the internal standard injected in 40 μl gave nearly a full-scale pen response on the W + W recorder Model 1200 set at 100 mV. The chart speed of the dual-channel recorder was 0.5 cm/min. The capacity factors (k') of the substances were bufuralol 4.7, carbinol metabolite 2.0, ketone metabolite 2.9, and internal standard 3.7. k' is defined as $(V_r - V_0)/V_0$, where V_r = retention volume and V_0 = void volume.

Extraction procedure

All glassware used in the assay was acid-washed in potassium dichromate in concentrated sulfuric acid, rinsed with deionized water and ovendried. There was no need of treatment with a siliconizing agent [2,3].

In a 12-ml glass-stoppered centrifuge tube there were added to 0.2 ml plasma (for concentrations below 25 ng/ml, 0.5 ml plasma) 0.2 ml of the following internal standard solution (prepared freshly each day); 200 μl (80 μl for samples with concentrations below 25 ng/ml) of the stock solution in methanol, containing 2.5 μg/ml, were pipetted into a 10-ml volumetric flask and diluted to volume with 2.5% NaOH. After mixing on a Vortex mixer, 5 ml of distilled diethyl ether were added, the tube was stoppered and rotated for 5 min on a rotary tube mixer. After centrifugation at 700 g, 4.5 ml of the organic layer were transferred by a glass pipette to another centrifuge tube, containing 1 ml of 0.1 N HCl. The extraction of the sample was repeated with a second aliquot of diethyl ether as described above. The combined ether extract was rotated for

5 min with the 0.1 *N* HCl. After centrifugation at 700 *g* the organic layer was discarded and 1 ml of diethyl ether added. After short mixing and centrifuging the ether was removed by aspiration. To the aqueous residue 0.2 ml of 1 *N* NaOH and 5 ml of dichloromethane were pipetted. The tube was rotated again for 5 min and centrifuged. The aqueous layer was aspirated and discarded; 4.5 ml of the organic phase were evaporated to dryness at 15—20°C with a vacuum evaporator (Rotavapor, Büchi, Flawil, Switzerland). The residue was dissolved in 100 µl of the mobile phase and an aliquot of 40 µl or 80 µl injected. The sample solutions were stored in a refrigerator. Parallel to the unknown samples, three plasma standards were analysed, according to the expected plasma levels (Table IV).

TABLE IV

AMOUNTS OF PLASMA AND PLASMA STANDARDS TO BE USED FOR THE DETERMINATION OF BUFURALOL

Expected concentration range of bufuralol (ng/ml)	Amount of plasma to be extracted (ml)	Amount of internal standard in 0.2 ml of 2.5% NaOH (ng)	Amount injected (µl)	Plasma standards to be used (No. from Table III)
≥25	0.2	10	80 40*	2, 3 and 4, if necessary 1 for high concentrations 5, 6, and 7, if necessary 4
<25	0.5	4	80	

*Plasma concentration above 100 ng/ml.

Calculations

The peak heights of the extracted plasma standards were directly proportional to the concentrations.

The plasma levels of the ketone metabolite were determined by calculating the linear regression curve, based on the extracted plasma standards and comparing the peak heights of the unknowns with this calibration curve.

The calculation of bufuralol and the carbinol were performed in two different ways:

(1) Using the peak-height ratio technique (ratio of the peak height of the compound to be determined to the peak height of the internal standard) a calibration curve was calculated as a linear regression with the extracted plasma standards. The unknown plasma levels were determined by interpolation of their peak-height ratio from these calibration curves.

(2) With a Hewlett-Packard data system 3353 connected parallel to the recorder (only the fluorescence measurements were considered), the unknown plasma concentrations were calculated, based on the peak-area ratio technique.

RESULTS AND DISCUSSION

Chromatographic system

The resolution of the chromatographic system was determined daily by injection of 40 µl of the reference mixtures, tabulated in Table I, in accordance

with the concentration range of the unknown plasma samples.

Chromatograms of plasma standards and unknown plasma samples are shown in Figs. 1 and 2. It is obvious that extracts of human blank plasma yielded no interferences from endogenous components in the fluorimetric determination. The UV detection of the ketone metabolite was more critical due to some variation of the baseline. The interpretation of the graphs was sometimes difficult near the limit of detection. Improved sensitivity should be expected with the recently available UV detectors.

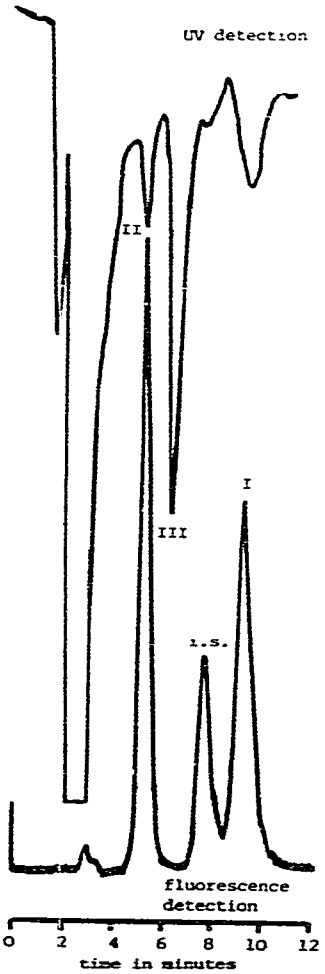


Fig. 1. Chromatograms of spiked plasma standards. Sample volumes of 0.2 ml of plasma were used: 10 ng internal standard in 0.2 ml of 2.5% NaOH were added, and 80 μl of the final solution were injected. Concentrations, in ng/ml plasma, were: bufuralol (I) 100; carbinol metabolite (II) 100; ketone metabolite (III) 200. Column: 250 mm × 3.2 mm, Spherisorb ODS, 5 μm. Mobile phase: methanol—0.02 *M* $(NH_4)_2HPO_4$ (pH 9.2) (75:25, v/v); flow-rate 0.6 ml/min. UV detection at 231 nm; fluorescence detection at 250 nm/300 nm.

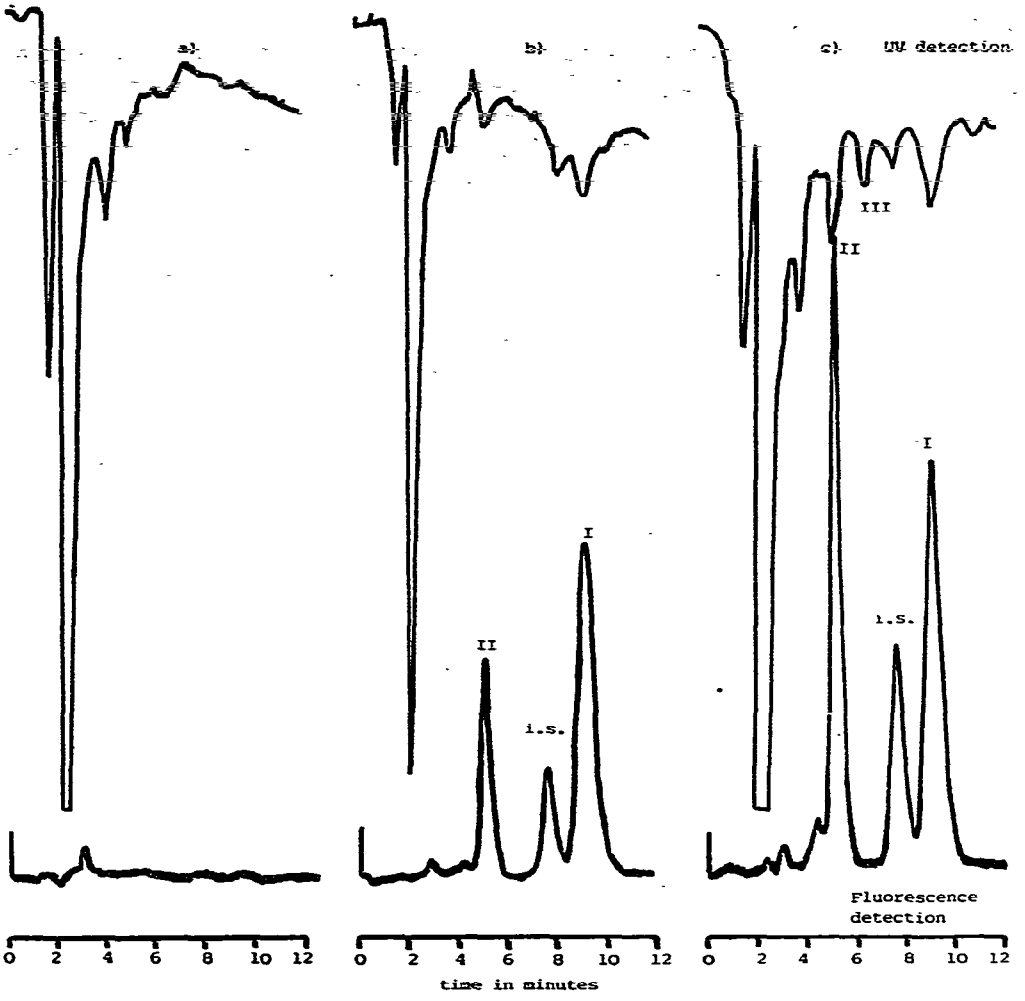


Fig. 2. Chromatograms of plasma extracts of a volunteer, who received an oral dose of 40 mg of bufuralol. Sample volumes of 0.2 ml of plasma were used; 10 ng internal standard in 0.2 ml of 2.5% NaOH were added. (a) Blank plasma, 80 μl injected. (b) 0.75 h after administration, 40 μl injected. (c) 5 h after administration, 80 μl injected. Bufuralol (I), carbinol metabolite (II), ketone metabolite (III), internal standard (i.s.). Test conditions were as given in Fig. 1.

Recovery

With the clean-up procedure described the overall extraction yields were at all concentrations as follows: bufuralol 72% ± 5%, carbinol metabolite 69% ± 6%, ketone metabolite 65% ± 4%, internal standard 68% ± 5%.

Sensitivity

The sensitivity of the HPLC method, using 0.5 ml of plasma, was 1 ng/ml for bufuralol and the carbinol metabolite, and 10 ng/ml for the ketone.

Relative standard deviation

The relative standard deviation of a single determination for the entire procedure, evaluated by analysing the same unknown plasma samples on different days, was found to be about ± 4—5% for bufuralol and the carbinol metabolite down to a concentration of 5 ng/ml. The corresponding relative standard deviation for the ketone metabolite could only be estimated; it was in the range of ± 10—20% at concentrations above 20 ng/ml.

Comparison of the results of the gas chromatography—mass spectrometry method with the HPLC method

In Table V plasma concentrations of the unchanged drug and the carbinol metabolite of a volunteer after a single oral dose of 40 mg of bufuralol determined by gas chromatography—mass spectrometry (GC—MS) and HPLC are compiled. The data show that the two methods give comparable results. Up to now not much information has been collected with respect to the determination of the ketone metabolite. However, it was found that after a single oral dose of 40 mg of bufuralol the concentration of the ketone metabolite is below 50 ng/ml within 24 h after the administration.

TABLE V

COMPARISON OF THE RESULTS OBTAINED WITH THE GC—MS METHOD* AND WITH THE HPLC PROCEDURE

Plasma concentrations are in ng/ml of the unchanged drug and the carbinol metabolite after a single oral dose of 40 mg of bufuralol (volunteer N.G.).

Time after administration (h)	GC—MS method		HPLC method (mean values)	
	Bufuralol	Carbinol metabolite	Bufuralol	Carbinol metabolite
0 (plasma)	<1	<1	<1**	<1**
0.25	23.8	15.9	22.8	15.4
0.50	210	87.7	210	85.6
0.75	210	88.8	200	88.8
1	78.7	55.2	73.2	55.1
1.5	215	99.7	197	100
2	196	103	181	97.8
3	168	101	159	105
4	57.7	57.5	59.6	61.8
5	97.9	100	100	85.7
6	65.7	91.9	64.5	86.2
8	40.2	77.5	33.5	68.4
10	22.2	61.1	—	—
12	14.3	49.5	14.2	42.8
24	2.3	14.9	1.6	12.8

*See ref. 3.

**No interferences.

CONCLUSIONS

The described HPLC method is rapid, sensitive and reproducible for the simultaneous determination of bufuralol and its carbinol metabolite. For pharmacokinetic studies, where large series of samples have to be analysed, this new procedure is easier to perform than the published GC—MS assay.

The ketone metabolite is also detectable, but the sensitivity and the reproducibility are poorer than for the two other substances.

The experience gained with bufuralol shows that, in extracts of plasma, the fluorimetric detection in HPLC is less sensitive to interfering endogenous components than the UV detection. The fluorimetric detection depends on two parameters, the emission and excitation wavelengths, yielding a higher specificity than the UV extinction. Generally, for pharmacokinetic studies of substances with native fluorescence it should be determined whether the fluorimetric detection assay may be more advantageous.

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