Journal of Chromatography, 222 (1981) 437—444
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 754

DETERMINATION OF PRENALTEROL IN PLASMA AND IN URINE BY GAS—LIQUID CHROMATOGRAPHY

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(First received July 25th, 1980; revised manuscript received October 6th, 1980)

SUMMARY

A description is given of a gas—liquid chromatographic method for the quantitative determination of unchanged prenalterol in plasma and for the total (free and conjugated) prenalterol in urine. After addition of an adequate internal standard, prenalterol together with the internal standard, is extracted with diethyl ether and derivatized with heptafluorobutyric anhydride—pyridine to form a tri-heptafluorobutyric derivative. This derivative has favourable properties for its estimation by gas—liquid chromatography using electron-capture detection. A large percentage of prenalterol is excreted as sulfate conjugate in man. Thus a hydrolysis step is added to the urine assay. The sensitivity of the method is about 2 ng/ml.

INTRODUCTION

Prenalterol, S-(—)-(4-hydroxyphenoxy)-3-isopropylamino-2-propanol hydrochloride, is a new potent β_1-stimulator [1]. Due to low oral doses, a sensitive assay procedure for the quantitative determination of prenalterol is required. The method described here is based on the gas—liquid chromatographic (GLC) assay as briefly outlined by Rönn et al. [2]. The assay procedure was optimized for high sensitivity and in addition a method for the determination of free prenalterol and prenalterol sulfate in human urine is given.

Hydrolysis of the sulfate conjugate of prenalterol, identified as the major metabolite by Hoffmann and Arfwidsson [3], may be achieved by enzymatic or chemical hydrolysis. The highly hydrophilic free prenalterol was extracted from plasma or urine with diethyl ether at alkaline pH.

Derivatization was carried out by pyridine-catalyzed acylation with hepta-

0378-4347/81/0000—0000/$02.50

fluorobutyric anhydride (HFBA) in diethyl ether. The resulting tri-HFB derivative has favourable properties for its GLC determination by electron-capture detection.

EXPERIMENTAL

Reagents

Prenalterol hydrochloride was obtained from Ciba-Geigy (Basle, Switzerland) and H 155/46, the internal standard from Hässle (Mölndal, Sweden). The buffer solution, pH 10.8 was made up from 2 mole Na_2CO_3 per litre and adjusted to pH 10.8 with 1 *N* hydrochloric acid. Heptafluorobutyric anhydride (HFBA), distilled, was purchased from Fluka (Buchs, Switzerland) and β-glucuronidase—arylsulfatase from Boehringer (Mannheim, G.F.R.). The column packing used was 3% OV-17 on Chromosorb W HP obtained from Supelco (Bellefonte, PA, U.S.A.).

Procedures

Unchanged prenalterol in plasma and urine. Plasma (1 ml) or urine (5—50 μl) (for calibration curves, addition of prenalterol as aqueous solution), 0.1 ml aqueous solution of internal standard (50 ng for range 2—50 ng/ml and 150 ng for range 20—200 ng/ml), 0.1 ml pH 10.8 buffer and 10 ml diethyl ether were shaken for 10 min at 200 rpm (on a mechanical horizontal shaker) and briefly centrifuged.

The organic phase and 0.5 ml 0.02 *N* sulfuric acid were shaken for 10 min at 200 rpm, briefly centrifuged and the organic phase was discarded by aspiration with a water pump. The aqueous phase, 0.1 ml pH 10.8 buffer and 10 ml diethyl ether were shaken for 10 min at 200 rpm, briefly centrifuged and then the organic phase was separated and evaporated to dryness under a stream of nitrogen at 40°C. To the dry residue were added 0.3 ml pyridine (1% solution in diethyl ether) and 0.1 ml HFBA, stoppered and left at room temperature (22°C) for 30 min. The reaction mixture was evaporated to dryness under a stream of nitrogen (ca. 20—30 min). The dry residue was dissolved in 2 ml toluene and washed with 1 ml water by shaking for 2 min followed by brief centrifugation. Aliquots of the organic phase (1—3 μl) were injected into the gas chromatograph.

Prenalterol sulfate conjugate in urine. Urine, 5—50 μl, diluted with water to 0.1 ml, and 0.1 ml 5 *N* hydrochloric acid were stoppered and heated for 90 min at 60°C in a water bath. The reaction mixture was evaporated to dryness under a stream of nitrogen at 40°C. To the dry residue were added 0.1 ml aqueous solution of internal standard (150 ng), 0.9 ml pH 10.8 buffer and 10 ml diethyl ether; the mixture was shaken for 10 minutes at 200 rpm, then the procedure as for plasma was carried out.

Gas chromatography

A Pye 204 instrument, equipped with a ^{63}Ni electron-capture detector, was used. Sample injection was carried out using an S8 Autojector.

A 2 m × 4 mm I.D. pyrex glass column, packed with 3% OV-17 on Chromosorb W HP (100—120 mesh), was used. The carrier gas was nitrogen at a flow-rate of 40 ml/min.

Temperatures were: column 195°C; injector 195°C; and detector 350°C. The retention times with these conditions were 2 min and 2.9 min for the derivatives of prenalterol and the internal standard, respectively.

RESULTS AND DISCUSSION

Extraction

The pH dependence of the extractability of prenalterol and internal standard (H 155/46) was evaluated by adjusting aqueous solutions to pH values ranging from 7—13, using dilute hydrochloric acid and sodium hydroxide. The samples were extracted with diethyl ether and derivatized with HFBA. The percentage extraction was estimated by comparison with pure HFB derivatives of both compounds. It was found that for both prenalterol and internal standard an extraction pH of 11 is optimal (Fig. 1).

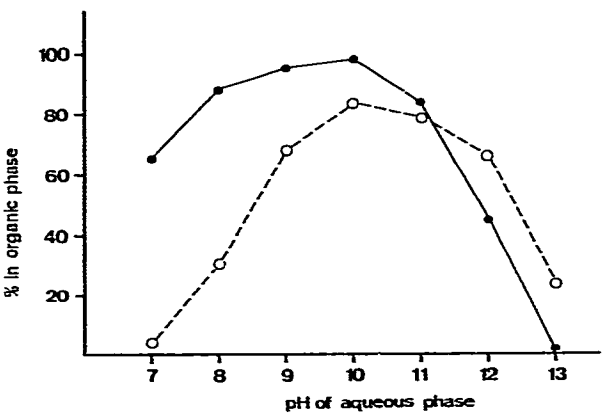
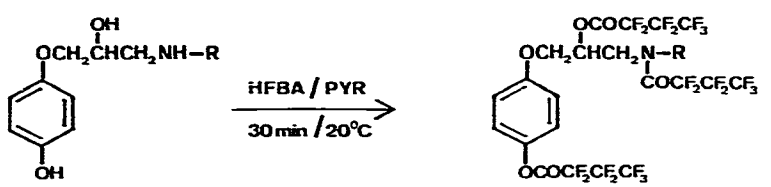


Fig. 1. Dependence of the extractability of prenalterol (○) and internal standard (●) on the pH values of the aqueous solution. Aqueous phase adjusted with 0.1 *N* hydrochloric acid or sodium hydroxide.

Derivatization

The acylation of prenalterol with HFBA results in satisfactory yields only if a catalyst (i.e. pyridine) is used (Fig. 2). After 15 min at room temperature the acylation reaction is already completed and remains unchanged for at least 3 h. There is no benefit if the reaction is carried out at a higher temperature. The



R : $CH(CH_3)_2$ = Prenalterol

R : $CH(CH_2CH_3)_2$ = int.standard (H 155/46)

Fig. 2. Structure of prenalterol, H 155/46 and their HFB derivatives.

yields of the derivatives are the same, but interfering background peaks increase dramatically. Thus an optimal reaction time of 30 min at room temperature was chosen. The structures of the HFB derivatives were verified by mass spectrometry.

Hydrolysis

For the determination of the sum of free and conjugated prenalterol in human urine, a hydrolysis step must precede the standard assay. About 50% of the prenalterol dose is excreted in the urine as a sulfate conjugate and ca. 20% as unchanged drug. Hydrolysis of the sulfate may be achieved either enzymatic-

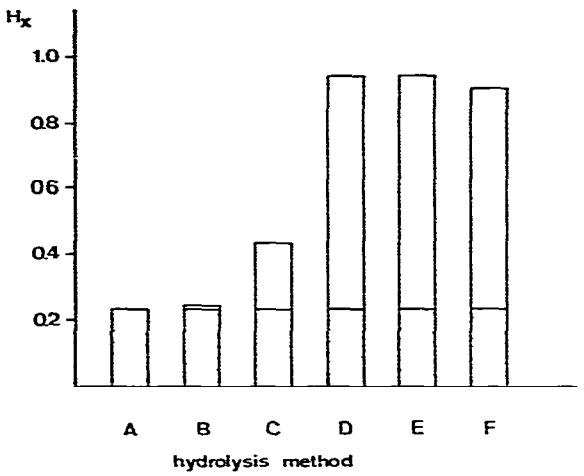


Fig. 3. Hydrolysis of the sulfate conjugate in a human urine sample (0—2 h after single oral dose of 10 mg prenalterol). (A) Free prenalterol (no hydrolysis); (B) total prenalterol after hydrolysis in 2.5 *N* hydrochloric acid for 1 h at 20°C; (C) total prenalterol after hydrolysis in 2.5 *N* hydrochloric acid for 16 h at 20°C; (D) total prenalterol after hydrolysis in 2.5 *N* hydrochloric acid for 1 h at 60°C; (E) total prenalterol after hydrolysis in 5 *N* hydrochloric acid for 1 h at 60°C; (F) total prenalterol after enzymatic hydrolysis with β-glucuronidase—arylsulfatase for 16 h at 37°C.

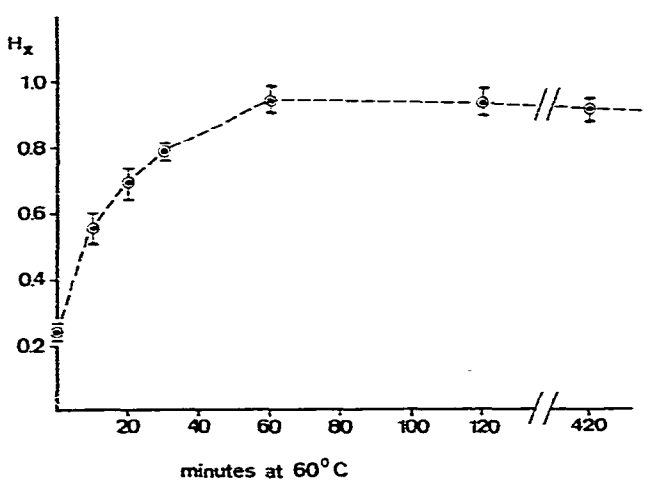


Fig. 4. Optimization of the acid hydrolysis. Human urine sample (0—2 h after a 10-mg dose of prenalterol) hydrolysed in 2.5 *N* hydrochloric acid at 60°C for up to 7 h.

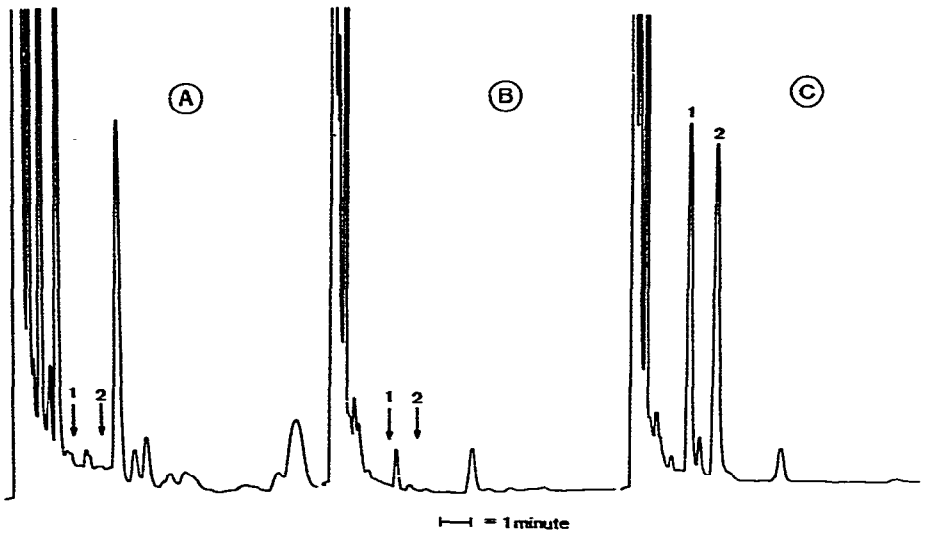


Fig. 5. Chromatograms of blank extract of 5 μl urine after (A) enzymatic hydrolysis; (B) acid hydrolysis; (C) extract of 5 μl urine from a volunteer (who had received a 10-mg dose of prenalterol), after acid hydrolysis, 150 ng internal standard added *after* hydrolysis. Peaks: 1 = prenalterol, 2 = internal standard.

ally or chemically by hydrochloric acid. A number of different hydrolysis conditions were tested using urine samples from a human bioavailability study. It was found that either the enzymatic method, using β-glucuronidase—arylsulfatase, or the chemical method, using 5 *N* hydrochloric acid, gave good results (Fig.3). Both methods were optimized and it was found that enzymatic hydrolysis was completed after 16 h at 37°C. Acid hydrolysis was completed after 1 h at 60°C (Fig.4). The two methods, compared by analysis of a human urine sample, gave the same results. However, the acid hydrolysis was the method of choice because a large number of background peaks were present in the chromatograms of the samples which had been treated with enzyme (Fig.5). Typical chromatograms of plasma extracts are shown in Fig.6.

Over-all yield

The absolute over-all yield (extraction from plasma and derivatization) was estimated to be about 55—60%. However, the use of an internal standard allows for recoveries of between 93.7 and 106.1%.

Calibration graphs

Calibration graphs for two concentration ranges in plasma were made. The low concentration range (5—50 ng/ml) is analysed with 50 ng internal standard and the higher concentration range (20—200 ng/ml) with 150 ng internal standard. The peak height ratios (H_x) as calculated by dividing the peak height of the prenalterol signal by the peak height of the internal standard signal are plotted in Fig.7.

Calibration graphs for urine are constructed with 150 ng internal standard because concentrations in urine are high. The urine graph has the same slope as the plasma graph for the higher range.

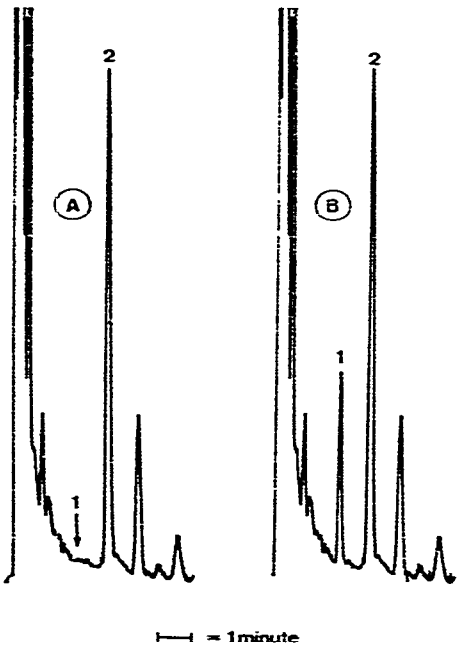


Fig. 6. Chromatograms of an extract of 1 ml plasma (A) containing 150 ng of internal standard; (B) spiked with 50 ng prenalterol and 150 ng internal standard. Peaks: 1 = prenalterol, 2 = internal standard.

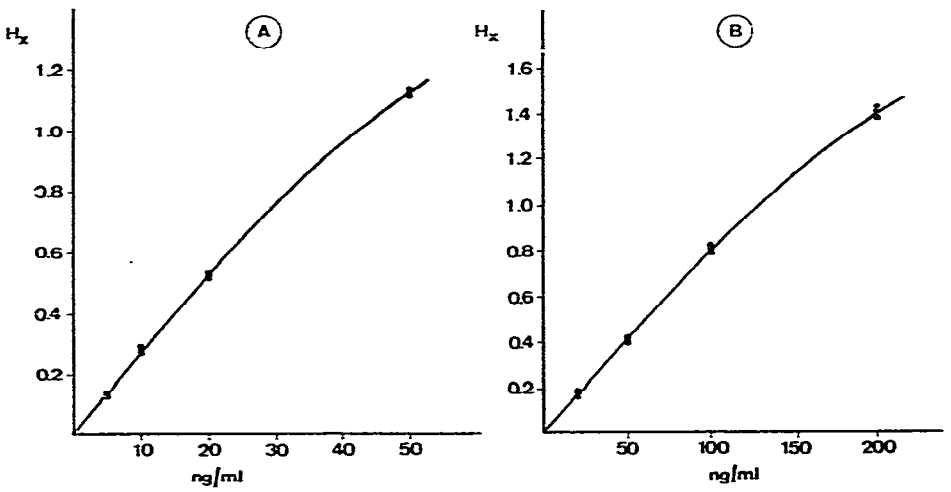


Fig. 7. Calibration graphs for the entire analytical procedure. (A) Low concentration range, with 50 ng internal standard; (B) high concentration range, with 150 ng internal standard.

Accuracy and precision

The accuracy and precision were evaluated by analysing spiked plasma and urine samples. The plasma samples contained 5.0—100 ng prenalterol per gram and the urine samples 0.175—3.735 μg prenalterol per gram.

Between ten and sixteen independent determinations were carried out with

TABLE I

TEST SAMPLES FOR METHOD VALIDATION

The spiked plasma and urine samples were analysed for prenalterol.

Initial	Found		*n*	% deviation of mean from initial
	$\bar{X}$	C.V. (%)		
Prenalterol in plasma (ng/g)				
5.0	5.0	20	16	0
45.0	44.0	5.5	16	−1.3
15.0	15.3	7.2	10	+2.2
100.0	97.0	2.8	10	−3.0
Prenalterol in urine (µg/g)				
0.175	0.175	9.1	3	0
0.555	0.575	2.6	3	+3.5
0.950	1.012	2.5	3	+6.1
2.082	1.979	1.6	3	−4.9
2.974	2.786	3.1	3	−6.3
3.735	3.571	1.1	3	−4.4

the plasma samples. The urine samples were analysed in triplicate. The coefficient of variation values ranged from 1.1 to 9.1% for all concentrations except the lowest (5 ng/g) where the coefficient of variation was 20%.

The deviation of the mean found from the given concentrations was between −6.3% and +6.1% (Table I).

Application

The applicability of these methods was tested by analysing plasma samples

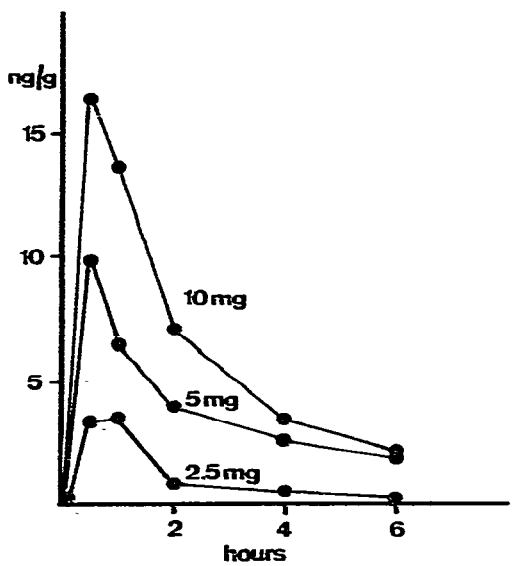


Fig. 8. Mean plasma levels of unchanged prenalterol in healthy volunteers ($n = 6$) following single oral doses of 2.5, 5 and 10 mg prenalterol.

from a dose—response study in healthy volunteers. Six volunteers received 2.5-, 5- and 10-mg single, oral doses of prenalterol (tablets) in a cross-over experiment. The mean plasma profiles are illustrated in Fig.8. The mean areas under the plasma curves were 6.9, 23.0 and 38.3 $ng \cdot g^{-1} \cdot h$ for the three dosages, respectively.

In a separate study, urine was collected quantitatively for 24 h following oral administration of 10 mg prenalterol (sustained release tablet) to one healthy volunteer. The free as well as the total (free and conjugated) prenalterol were measured using the described methods. The following amounts were excreted: A, free prenalterol, 2.16 mg/24 h (21.6% of the dose); B, total prenalterol, 7.47 mg/24 h (74.7% of the dose); C, (B − A) conjugated prenalterol, 5.31 mg/24 h (53.1% of the dose).

ACKNOWLEDGEMENTS

Thanks are due to Dr. P. Imhof for providing the biological samples and to Mr. P. Zbinden, Mr. B. Keller and Miss A. Sieber for expert technical assistance in optimizing the procedures.

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