Journal of Chromatography, 162 (1979) 439 445
Biomedical Applications
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 277

Note

Quantitative analysis of naphthoxylactic acid, a major metabolite of propranolol in plasma in man

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(Received June 14th, 1978)

To be able to understand fully the multiplicity of actions produced by the beta-adrenergic receptor blocking drug propanolol in man it is important to understand its metabolism and disposition. Thus, as more than 98% of the drug is metabolized following oral administration in man [1, 2], it is possible that metabolites may contribute to its actions. It has already been shown that 4-hydroxypropranolol, equipotent with propranolol as a beta-blocker [3], can be present in plasma in similar concentrations to its parent compound in patients chronically treated with propranolol [4].

Although the major metabolite of propranolol in human urine has been indicated to be naphthoxylactic acid (NLA) [5—7], no information is available on its plasma concentrations, in particular after chronic propranolol therapy.

The present study describes a sensitive and specific technique for NLA determinations in plasma by electron capture gas chromatography (GC) following methylation of the carboxyl group with diazomethane and acylation of the hydroxyl group with heptafluorobutyric anhydride. The method was applied to quantitative determinations in the plasma of patients chronically treated with propranolol.

EXPERIMENTAL

Standards and reagents

Naphthoxylactic acid (NLA) was kindly supplied by I.C.I. (Macclesfield, Great Britain).

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Trimethylamine was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) and was dissolved in nanograde benzene. Nanograde benzene was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Diazomethane was prepared from N-methyl-N-nitroso-*p*-toluene-sulfonamide in diethyl ether using a Diazald® kit from Aldrich (Milwaukee, Wisc., U.S.A.). Heptafluorobutyric anhydride was obtained from Pierce (Rockford, Ill., U.S.A.) in 1-ml ampules and was stored in airtight PTFE-capped 3-ml vials (Reacti-Vials, Pierce).

Aqueous reagents, including pH 6.0 phosphate buffer, 0.5 *M*, and hydrochloric acid, 1 *M*, were prepared from glass-distilled water and stored in glass bottles with PTFE-lined caps.

Synthesis

The ethyl ester of NLA, used as the internal standard, was prepared by extractive alkylation. To 50 mg of NLA ($2 \cdot 10^{-4}$ mole) in a 15-ml centrifuge tube were added 0.75 ml of 10% tetrabutylammonium hydroxide in water ($3 \cdot 10^{-4}$ mole), 30 μl ethyl iodide ($3 \cdot 10^{-4}$ mole) and 4 ml of methylene chloride. The mixture was shaken vigorously for 1 h and was then centrifuged. The top, aqueous, layer was removed and the methylene chloride was evaporated at 50° under a stream of nitrogen. To the residue was added 5 ml of benzene. After washing with 2 ml of pH 6.0, 0.5 *M*, phosphate buffer the benzene was evaporated to dryness. A white powder was obtained, melting point 49—51°. The ethylation was quantitative as confirmed by gas chromatography—mass spectrometry (GC—MS).

Glassware

All glassware was cleaned in chromic acid. Conical and round-bottomed glass centrifuge tubes were silanized as previously described [8].

Instruments

GC. A Varian Model 1440 gas chromatograph equipped with a ^{63}Ni electron capture detector was used. The column (180 cm × 2 mm I.D.) was made of pyrex glass and packed with 80—100 mesh Chromosorb W coated with 5% OV-17. The column was conditioned at 250° for 48 h before use. Operating conditions were: column temperature 185°, injector temperature 265°, detector temperature 270°, and carrier gas (nitrogen) flow-rate 30 ml/min.

GC—MS. The combination instrument was an LKB 9000S, operated at an accelerating voltage of 3.5 kV, ionization voltage of 20 eV, and a trap current of 60 μA. A pyrex column (120 cm × 2 mm I.D.) with 4% OV-17 at 190° was employed. The carrier gas (helium) flow-rate was 20 ml/min.

Patients

Blood samples were obtained 2 h after the morning dose from seven hypertensive patients chronically treated with propranolol, 40 mg every 6 h, and were studied in a clinical research unit [9]. The blood samples were collected in heparinized tubes and immediately centrifuged at 6000 *g* for 15 min in a refrigerated centrifuge and the clear plasma layer removed. The plasma samples were either analyzed immediately or stored at —70° in an ultra-cold freezer.

Extraction and derivatization

A 1.00-ml plasma sample was acidified to ≈ pH 2 with 0.2 ml of 1 *N* hydrochloric acid in a 15-ml round-bottomed centrifuge tube. After the addition of the internal standard, 400 ng, the sample was extracted on a reciprocating shaker for 10 min. The organic phase was transferred to a conical centrifuge tube and evaporated to dryness in a water bath at 50—60° under a gentle stream of nitrogen.

Methanol, 100 μl, and the diethyl ether solution of diazomethane, 200 μl, were added and allowed to react for 10 min at room temperature. The excess reagent was evaporated as described above. A 400-μl volume of nanograde benzene was then added, together with 25 μl of 1 *M* trimethylamine in benzene and 50 μl of heptafluorobutyric anhydride. The tubes were capped and heated at 50° for 10 min. The reaction mixture was then washed with 1 ml of pH 6.0 phosphate buffer, 0.5 *M*, by vigorous shaking for 30 sec. After centrifugation, 0.5—4 μl of the benzene layer was injected.

Standard curves

Varying quantities of synthetic NLA (100—1000 ng) and 400 ng of the internal standard were added to 1.0-ml samples of control human plasma and carried through the analytical procedure. Peak area ratio measurements times the concentration of the internal standard were plotted against the concentration of NLA. Synthetic NLA was also extracted from 1.00-ml samples of distilled water as well as derivatized without extraction to obtain an index of recovery.

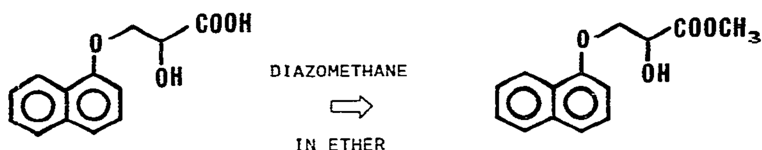
Propranolol was determined by a specific GC—MS assay [10] recently modified to include stable-isotope-labeled propranolol as the internal standard [11].

RESULTS AND DISCUSSION

The two-step derivatization procedure employed for NLA is depicted in Fig. 1. Both reactions proceed rapidly and quantitatively to yield the methyl,heptafluorobutyryl derivative, the structure of which was confirmed by GC—MS, Fig. 2A. A similar derivatization technique has been applied to acidic catecholamine metabolites [12]. The acylation step in the present study followed a procedure previously shown to lead to rapid acylation of a variety of compounds [13, 14], including propranolol [8] and several of its basic and neutral metabolites [10, 15]. Excess reagents were removed by a pH 6.0 buffer wash similar to the method of previous studies [8, 10, 14]. This procedure resulted in a minimum of interferences on the electron capture detector. The derivative was stable for more than 48 h in contact with the buffer.

While the methylation step was necessary in order to obtain good peak symmetry, the acylation of the hydroxyl group with heptafluorobutyric anhydride provided a unique tool to enhance the electron capture sensitivity of NLA specifically as compared with interfering endogenous carboxylic acids not containing a hydroxyl group. The minimum detectable quantity of the methyl, heptafluorobutyryl derivative of NLA was about 2 pg, which is similar to other heptafluorobutyryl esters [13].

STEP 1:



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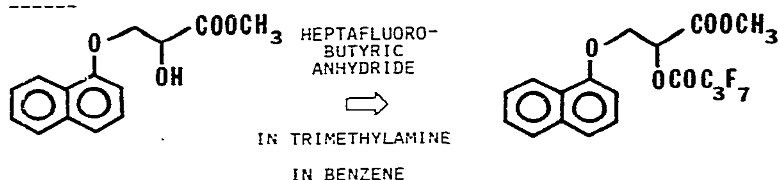

Fig. 1. Derivatization procedure for NLA.

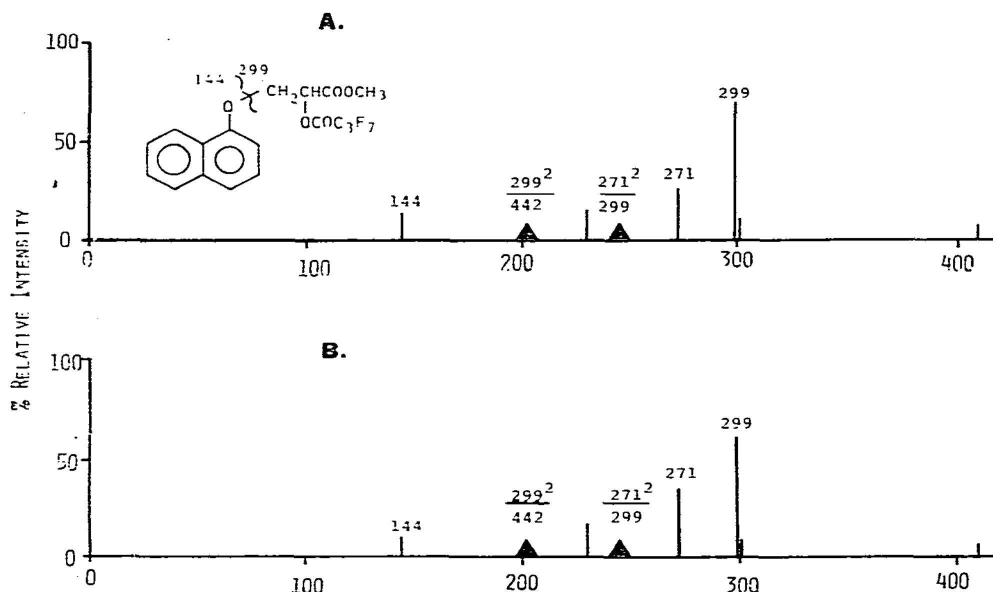

Fig. 2. Mass spectra of the methyl,heptafluorobutyryl derivative of NLA. (A) Synthetic NLA; (B) propranolol metabolite in plasma.

The chromatogram of a control plasma extract is shown in Fig. 3A, demonstrating no interference at the retention time of NLA and only a minor interference at the retention time of the internal standard, the ethyl ester of NLA. Fig. 3B shows the chromatogram of an extract of plasma from a patient chronically treated with propranolol, 160 mg/day, to which the internal standard, 400 ng/ml, had been added. The high NLA concentration (656

TABLE I

PLASMA LEVELS OF NLA AND PROPRANOLOL IN SEVEN PATIENTS ON CHRONIC PROPRANOLOL THERAPY, 160 mg PER DAY

All samples were collected at 2 h after the morning dose, i.e. at the time for peak plasma propranolol [9].

Patient	Propranolol* (ng/ml)	NLA (ng/ml)	NLA Mean ±S.D.	Ratio NLA : Propranolol
I	46	660 632 676	656 ±22	14.5
II	63	536 500 496	510 ±22	8.1
III	53	917 960 920	932 ±24	17.6
IV	51	365 320 365	350 ±26	6.9
V	45	540 545 521	535 ±13	11.9
VI	38	405 400 365	390 ±22	10.3
VII	100	460 510 450	473 ±32	4.7

*Determined as part of a separate study (see ref. 9).

ng/ml) present in this patient at this moderate propranolol dose indicates that the method developed is quite adequate for determinations of this propranolol metabolite in man. At these concentration levels the minor interference with the internal standard seen in Fig. 3A becomes insignificant. The high NLA concentration present also made it possible to obtain a complete mass spectrum of this metabolite in the plasma of this patient, Fig. 2B. This spectrum is identical to the mass spectrum of the synthetic material, Fig. 2A.

The standard curve for NLA in plasma was linear for the concentration range studied (100—1000 ng/ml) and went through the origin (slope 1.04; correlation coefficient >0.99). Extraction from water as well as derivatization without prior extraction gave the same slope, indicating no adverse effect due to the extraction. When the internal standard was added to the NLA plasma samples after the extraction but prior to the derivatization, a 20% lower value was obtained, indicating that the recovery of NLA in the extraction step was about 80%. The minimum detectable plasma concentration of NLA was about 2 ng/ml.

Table I summarizes the plasma concentrations of NLA measured in seven patients chronically treated with propranolol, 160 mg/day. Triplicate samples were measured for each patient. The NLA concentrations are expressed as the mean ± S.D. of these determinations. The table also gives the propranolol concentrations as a comparison. The specificity of the method is emphasized by the fact that no interferences were observed from several other drugs concomitantly administered to these patients. These drugs were clofibrate, digoxin, chlordiazepoxide, nitroglycerin, isosorbide dinitrate, tolazamide and warfarin.

The NLA concentrations in the seven patients ranged from 350 to 930 ng/ml, with a reproducibility for the individual patients of between 2.4 and 7.5%. These concentrations exceeded the propranolol concentrations measured in the same patients by five to eighteen times. There was no apparent relationship between the NLA and propranolol concentrations observed except for a similar, only 2.6-fold, between-patient variation in these concentrations, which supports the view [9] of only small between-patient variations in propranolol pharmacokinetics.

Although no pharmacological activity has been described for NLA, the high concentrations of this propranolol metabolite observed in plasma warrant further studies of both its pharmacokinetics and possible biologic activity.

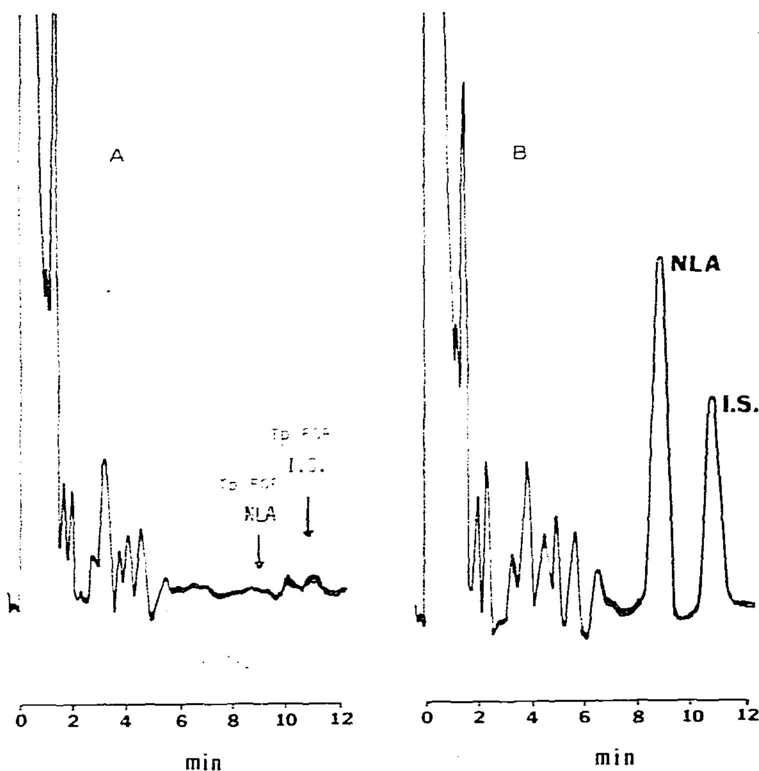

Fig. 3. Gas chromatograms of derivatized plasma extracts using electron capture detection. (A) Control plasma; (B) plasma from patient chronically treated with propranolol, 160 mg/day. Internal standard = ethyl ester of NLA, 400 ng/ml. NLA calculated = 656 ng/ml.

Furthermore, as lactic acid metabolites have been indicated to represent an important metabolic pathway for other beta-blockers [16—18], such studies extended to beta-blocking drugs in general may be important.

ACKNOWLEDGEMENTS

We thank Kristina Walle for excellent technical assistance and for her help in preparing the manuscript. This work was supported by grants GM 20387 and RR-01070 of the National Institute of General Medical Sciences.

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