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A THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ACEBUTOLOL AND ITS MAJOR METABOLITE IN SERUM

J. M. STEYN

Department of Pharmacology, University of the Orange Free State, Bloemfontein 9301 (South Africa)

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SUMMARY

A sensitive and specific thin-layer chromatographic method for the simultaneous determination of acebutolol [DL-1-(2-acetyl-4-*n*-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane] and its major metabolite [DL-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane] is described. A 2-ml volume of serum with 350 ng of quinidine as internal standard was extracted at pH 10, the solvent was evaporated off and the residue was dissolved in 50 μl of methanol. A 10-μl volume of the solution was spotted on a thin-layer plate and after elution (ethyl acetate–methanol–ammonia, 75:20:5) the plate was dried at 90° for 15 min and, after cooling, dipped in a 10% paraffin wax solution. The fluorescence was measured using a spectrofluorimeter with a thin-layer scanning attachment. The peak-height ratios of acebutolol to internal standard and metabolite to internal standard were used to quantitate acebutolol and the metabolite, respectively.

INTRODUCTION

Acebutolol is a β-adrenoceptor blocking agent whose pharmacological effects have been investigated[1–10], but no method for its determination in serum or urine has been published. The design of a clinical trial protocol to evaluate the antihypertensive properties of acebutolol necessitated its determination in serum.

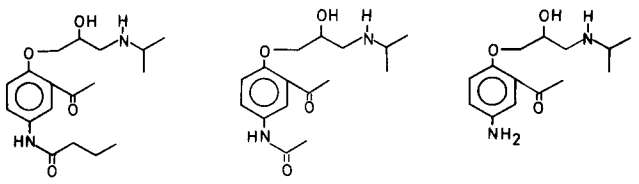
This paper describes a thin-layer chromatographic (TLC) procedure for the simultaneous determination of acebutolol (I) and its major metabolite (II) (Fig. 1) in biological fluids using quinidine as an internal standard.

The method is easy to perform, reproducible, requires very little handling and is therefore suitable for the routine determination of this drug in situations where serum levels may have to be determined in order to establish the desired therapeutic dose for a particular patient.

MATERIALS AND METHODS

Reagents

The reagents and solvents used were of guaranteed reagent grade (Merck, Darmstadt, G.F.R.).



I II III

Fig. 1. Structures of acebutolol [DL-1-(2-acetyl-4-*n*-butyramidophenoxy-2-hydroxy-3-isopropylaminopropane] I, its major metabolite [DL-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane] II, and their hydrolysis product [DL-1-(2-acetyl-4-aminophenoxy)-2-hydroxy-3-isopropylaminopropane] III.

Buffer solution

A buffer solution of pH 10 was prepared by adding 0.2 *M* sodium carbonate solution to 0.2 *M* sodium hydrogen carbonate solution until the required pH was reached.

TLC developing solvent

This solvent was freshly prepared before use and consisted of ethyl acetate–methanol–ammonia (75:20:5).

Apparatus

A Perkin-Elmer MPF 3 spectrofluorimeter equipped with a thin-layer scanning attachment was used to measure the fluorescence of the spots. The following operating conditions were used: light source, xenon lamp; excitation wavelength, 350 nm; emission wavelength, 450 nm; excitation slit, 10 nm; emission slit, 3 nm; scanning speed, "high" (4 cm/min); and paper speed, "low" (2.5 cm/min). The emission slit width, amplifier sensitivity, zero suppression and sample adjustment were set so as to obtain about 80% of full-scale deflection on the recorder when the highest concentrations on the plate were being measured.

Thin-layer chromatographic equipment

Pre-coated silica gel 60 TLC plates with a layer thickness of 0.25 mm and without a fluorescent indicator were used (Merck). The final solutions were applied to the plate by means of 5-μl disposable glass micropipettes (Clay-Adams, Division of Becton Dickenson, Parsippany, N.J., U.S.A.). The TLC plates were developed in a Shandon S/P Chromatank (Shandon Scientific, London, Great Britain).

Stock solutions

Stock solutions of acebutolol and the metabolite were prepared by weighing accurately (on a Mettler ME 22 microbalance) sufficient of the hydrochloride salts of these two compounds to be equivalent to 10 and 1 mg of the free compounds, respectively, and then dissolving in 10 ml of distilled water. These solutions were diluted so as to obtain solutions containing 1 μg per 10 μl and 100 ng per 10 μl, respectively.

Quinidine·HCl equivalent to 10 mg of free base was weighed as described above and dissolved in 10 ml of distilled water. From this stock solution, a solution

containing 10 ng of quinidine as free base per microlitre of water was prepared by 100-fold dilution.

These solutions were found to be stable for at least 1 month when stored at 0–4°.

Dipping solution

A 200-g amount of paraffin wax (m.p. 42–44°) was dissolved in 2 l of light petroleum (b.p. 40–60°).

Control solution

A solution containing 200 ng of acebutolol and 50 ng of quinidine per 5 μl of methanol was prepared and used to adjust the slope of the standard graph of acebutolol concentration *versus* acebutolol:quinidine peak-height ratio.

Extraction procedure

To 2 ml serum were added 350 ng of quinidine as internal standard and 2 ml of buffer (pH 10). The mixture was extracted once with 5 ml of ethyl acetate with gentle shaking on a mechanical shaker for 15 min. After centrifuging, the ethyl acetate extract was transferred to a conical tube, evaporated under a stream of nitrogen at 50° and the residue was dissolved in 50 μl of methanol. Care was taken to rinse the sides of the tube with methanol so as to ensure complete dissolution of the substance.

Spotting the plates

A 10-μl volume of the methanol solution was spotted on a thin-layer plate in two equal portions. Between applications, the spot was dried with a hair-drier. The liquid was allowed to run on the plate by gravity and the natural capillary action of the plate only.

Treatment of the TLC plate

Separation of the drugs was effected by ascending chromatography in an unsaturated TLC chamber at room temperature, the solvent being allowed to migrate to a height of 5 cm. The plate was then removed from the chamber, dried with a hair-drier and heated in an oven at 90° for 15 min. The plate was allowed to cool to room temperature and then dipped in the 10% (w/v) solution of paraffin wax in light petroleum and dried with a hair-drier, scanning being performed 15 min later.

RESULTS AND DISCUSSION

The technique of using the fluorescence of drugs on thin-layer plates for their determination is becoming increasingly popular and has been applied in a number of instances[11–14]. This procedure, however, is usually based on measurement of the fluorescence of the drug contained in a specific amount of serum against the fluorescence of standards made up in serum and treated in the same way as the sample to be analyzed. The peak height of the drug of unknown concentration can then be converted into a concentration value by relating it to the peak heights of the drugs of known concentration. The use of an internal standard to quantitate a drug by means

of fluorescence spectroscopy by using direct scanning of a thin-layer plate is fraught with a number of problems:

(a) the R_F value of the internal standard must be such that it does not interfere with the compounds to be determined (Table III);

(b) the fluorescence characteristics of the internal standard must be of such a nature that its excitation and emission wavelengths will be of the same order as those of the compounds to be determined;

(c) if the compounds to be determined are naturally fluorescent, then the internal standard must also exhibit natural fluorescence or, if the compounds to be determined can be induced to exhibit fluorescence, then the internal standard must be of such a nature that it will also be fluorescent when treated in the same way.

In this instance, we were fortunate to be able to use quinidine, as it appears at a convenient position on the TLC plate and exhibits natural fluorescence in the same region as acebutolol.

Effect of time on fluorescence of acebutolol and quinidine

We noticed that time had a pronounced effect on the fluorescence of acebutolol and, to a lesser extent, on that of quinidine. This effect is illustrated by the peak-height ratio of acebutolol to quinidine determined at 10-min intervals during a period of 4 h (Fig. 2), and appears to be due to the presence of trace amounts of ammonia on the plates, as the decrease in the fluorescence of acebutolol could be duplicated during a much shorter period by heating the plate in an oven to remove all traces of ammonia. This procedure, however, decreased the fluorescence of acebutolol to such an extent that the determination of low concentrations became very difficult and unreliable.

Effect of dipping the plates in a paraffin wax solution

It was found that by dipping the plates in a 10% (w/v) solution of paraffin wax in light petroleum, after a period of heating to remove ammonia, fluorescence

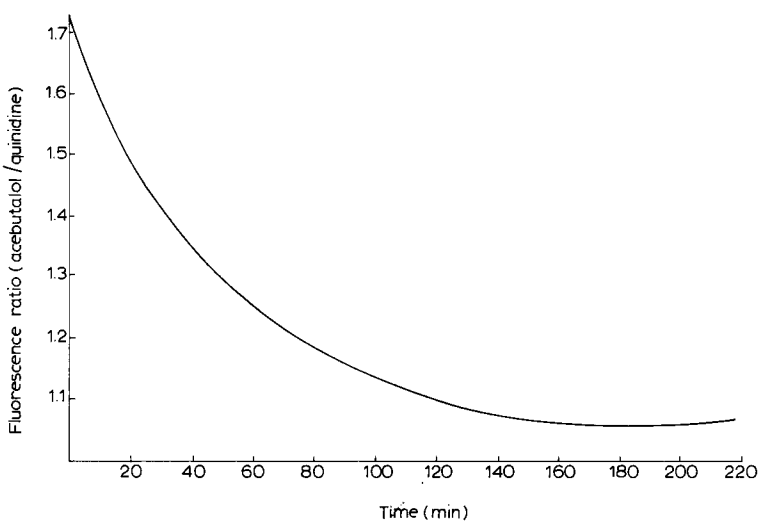


Fig. 2. Effect of time on acebutolol:quinidine fluorescence ratio.

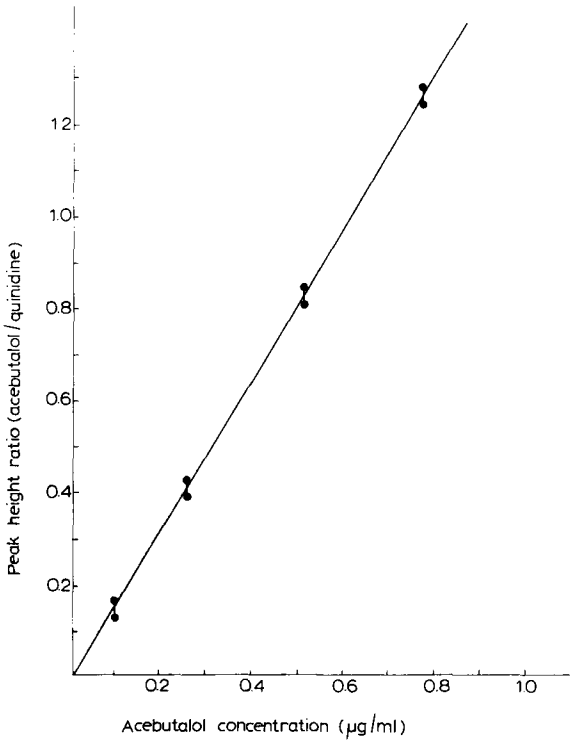


Fig. 3. Calibration graph for the quantitation of acebutolol and its main metabolite.

of all of the compounds involved could be stabilized for at least 48 h. The dipping procedure was also found to increase the fluorescence of the different compounds about 8-fold. The heating time before the plate was dipped did not appear to be crucial. We standardized the method by heating for 15 min at 90° and cooling for 5 min. This method ensured excellent reproducibility.

Quantitation of acebutolol

By adding a specific amount of internal standard (350 ng) and acebutolol in concentrations ranging between 0.1 and 1.0 μg/ml to drug-free human serum, extracting the serum according to the above procedure and measuring the fluorescence of the different compounds, a graph was constructed of acebutolol concentration *versus* acebutolol:internal standard peak-height ratio (Fig. 3). For determining an unknown concentration, the peak-height ratio was calculated and the concentration determined by interpolation from the standard graph.

Use of control solution to adjust slope of standard graph

At the time of constructing the standard graph of acebutolol concentration *versus* acebutolol:quinidine peak-height ratio, 5 μl of the control solution were spotted on the plate and the acebutolol:quinidine peak-height ratio was calculated. This ratio was then used to adjust the slope of the standard graph when the acebutolol concentration in a patient's serum was determined.

Example. Peak-height ratio of control solution when standard graph was constructed = 1.50; slope (concentration/peak-height ratio) = 0.63. When determining acebutolol, the control solution was spotted and the peak-height ratio noted.

Peak height ratio (control) = 1.60

$$\text{Adjusted slope} = \frac{1.50}{1.60} \cdot 0.63$$

$$= 0.59$$

This adjusted slope was then used to calculate the unknown concentration.

Quantitation of the metabolite

We found that the metabolite exhibited the same extraction and fluorescence characteristics as acebutolol. By calculating the peak-height ratio of metabolite to internal standard, the standard graph that is used to quantitate acebutolol can also be used to quantitate the metabolite (Fig. 3).

Reproducibility

By analyzing different concentrations of acebutolol in quadruplicate, spotting each concentration in duplicate and determining the peak-height ratios, the method was shown to exhibit excellent reproducibility. The results are presented in Table I.

TABLE I

REPRODUCIBILITY OF EXTRACTION PROCEDURE

$y = 0.633285\ x - 0.009656$. Correlation coefficient = 0.9958.

Acebutolol concentration (μg/ml)	*Ratio:* $\frac{\text{acebutolol peak height}}{\text{internal standard peak height}}$	*Mean ± S.D.*
0.1	0.17	0.165 ± 0.006
0.1	0.17	
0.1	0.16	
0.1	0.16	
0.25	0.41	0.42 ± 0.008
0.25	0.42	
0.25	0.43	
0.25	0.42	
0.5	0.79	0.813 ± 0.003
0.5	0.78	
0.5	0.83	
0.5	0.85	
0.75	1.18	1.19 ± 0.042
0.75	1.18	
0.75	1.15	
0.75	1.25	

Recovery of acebutolol from serum

After preparation of the standard graph, sera containing specific amounts of acebutolol were prepared blind, and given to an analyst to determine the concentrations. The results were extremely satisfactory (Table II).

TABLE II

RECOVERY OF ACEBUTOLOL FROM SERUM

Amount of acebutolol added (μg/ml)	*Amount recovered (μg/ml)*	*Mean ± S.D. (μg/ml)*
0.32	0.30	0.308 ± 0.005
	0.31	
	0.31	
	0.31	
0.62	0.62	0.61 ± 0.008
	0.61	
	0.60	
	0.61	

TABLE III

R_F VALUES OF DRUGS INVESTIGATED

Compound	R_F	*Compound*	R_F
Acebutolol	0.56	Sotalol	0.54
Metabolite	0.66	Oxprenolol	0.7
Quinidine	0.40	Alprenolol	0.74
Propranolol	0.74	Prindolol	0.68
Practolol	0.60		

Specificity

The β-adrenoceptor blocking agents propranolol, practolol, sotalol, oxprenolol, alprenolol and prindolol were tested for interference, but it was found that none of them interfered as they did not exhibit fluorescence and had R_F values different from those of the compounds to be determined (Table III).

Investigation of other methods for the determination of acebutolol

Neither acebutolol (I) nor its metabolite (II) was found to be suitable for gas chromatography, as they are very labile under gas chromatographic conditions. Derivatization (acetylation and silylation) resulted in the formation of the desired derivatives, but is was found that these compounds were very labile and hydrolyzed too readily to be of any use.

Hydrolysis of acebutolol and its metabolite with 3 *M* sulphuric acid to the corresponding arylamine (III) (Fig. 1), followed by diazotization and reaction wih naphthylethylenediamine, is a means of determination by UV absorption[5]. However, this method is not selective because it does not discriminate between the drug and its metabolite, nor is it specific as a number of drugs may have similar UV absorption characteristics and could interfere (*e.g.*, propranolol).

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