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OPTIMIZATION OF FLUORESCENCE DETECTION FOR THE DETERMINATION OF LABETALOL IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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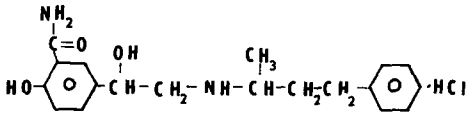
SUMMARY

A sensitive reversed-phase high-performance liquid chromatographic method for the determination of labetalol has been developed. A mobile phase consisting of citrate buffer (pH 6.5), acetonitrile and 2-propanol and an RP-8 column were used. The sensitivity of the fluorescence detection was enhanced to 1 ng/ml of labetalol in plasma by optimizing the emitted light. General guidelines for optimization of fluorescence detection are discussed.

INTRODUCTION

Labetalol, 5-{1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl}salicylamide hydrochloride (Fig. 1), is an antihypertensive agent, and its determination in human plasma after a single 200-mg oral dose requires an analytical method sensitive to 10 ng/ml.



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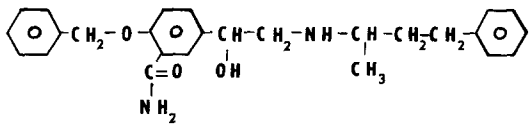

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Fig. 1. Structures of labetalol hydrochloride (I) and the internal standard, *o*-benzyllabetolol (II).

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Selective and sensitive high-performance liquid chromatographic (HPLC) methods for labetalol have been reported[1–4]. The recent literature on the determination of labetalol in plasma was summarized by Alton *et al.*[5]. The main problem in the determination of labetalol is the high pH of the mobile phase; a pH of *ca.* 9.5 has been found necessary to achieve sufficient sensitivity of fluorescence detection[1,5], which is too high for the common silica-based HPLC columns. Therefore, Alton *et al.*[5] proposed the use of the spherical macroporous copolymer RP-1, which permits operation in alkaline medium. Recently, the combination of a solid-phase extraction module on-line with thermospray HPLC–mass spectrometry, with a limit of detection of *ca.* 5 ng/ml, was reported[6].

However, a method employing a more common silica-based reversed-phase column would be highly desirable. The aim of this paper is to suggest that optimization of fluorescence detection may enhance the sensitivity at neutral or even slightly acidic pH sufficiently to make separation on a common alkylsilica reversed-phase column feasible.

EXPERIMENTAL

Both labetalol hydrochloride and the internal standard (*o*-benzyllabetalol, Fig. 1) were prepared at the Drug Research Institute, Modra, Czechoslovakia. Labetalol was checked against the commercially available product (Allen and Hanburys, London, U.K.). Acetonitrile (UV grade) was obtained from Merck (Darmstadt, F.R.G.) and 2-propanol (UV grade) from Lachema (Brno, Czechoslovakia). All other chemicals were of analytical-reagent grade from Lachema.

A Spectra-Physics (Darmstadt, F.R.G.) Model SP 8100 liquid chromatograph was used, in combination with a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 1000 M fluorescence detector. The excitation light was filtered through a 311-nm interference filter (Balzers, Vaduz, Liechtenstein). Either a 400-nm interference filter (Balzers) or a 400-nm long-pass filter (Carl Zeiss, Jena, G.D.R.) was used as an emission filter.

The injection valve was equipped with a 100-μl sample loop. A Spectra-Physics SP 4200 computing integrator was used to evaluate the fluorescence signal.

Separations were performed on a LiChrosorb 5 RP-8 column (250 × 4.6 mm I.D.) from Spectra-Physics. The citric acid mobile phase was prepared by mixing 50 parts of 0.05 *M* sodium citrate solution (adjusted to pH 6.5 with 1 *M* citric acid) with 28 parts of 2-propanol and 22 parts of acetonitrile. Sodium oxalate (pH 7.0) and tartrate (pH 7.0) mobile phases were prepared in an analogous manner. The system was operated at ambient temperature and at a flow-rate of 0.8 ml/min.

A 2-ml volume of human plasma was mixed with 50 μl of an aqueous solution of the internal standard (100 μg/ml) and 200 μl of pH 9 buffer (90 g of sodium hydrogencarbonate and 22.0 g of potassium carbonate per litre. The mixture was extracted with 4 ml of diethyl ether. After separation of the phases by centrifugation, the extraction was repeated with 3 ml of diethyl ether. The combined ether layers were reduced to dryness *in vacuo* at 40°C, the residue was dissolved in 400 μl of acetonitrile–water (1:1) and 100 μl were injected into the liquid chromatograph.

RESULTS AND DISCUSSION

For the optimization of the fluorescence sensitivity, the common practice is to reduce the fluorescence signal of the mobile phase, which is achieved by filtration of the emitted light. Thus, the variable to be optimized is the signal-to-noise ratio, H/N_H:

$$H = S - G \tag{1}$$

where H = effective signal, S = fluorescence of the sample and mobile phase, G = fluorescence of the mobile phase background and N_H = noise of H.

Based on the theory of fluorescence spectra, the noise of the signal, N_S, is

$$N_S = k\sqrt{S} \tag{2}$$

where k is a proportionality constant. The noise of the background fluorescence, N_G, is

$$N_G = k\sqrt{G} \tag{3}$$

Using the error propagation theory, the noise of the effective signal becomes

$$N_H^2 = N_G^2 + N_S^2 \tag{4}$$

Combining eqns. 1–4, we obtain the following expression for the signal-to-noise ratio of the effective signal, H:

$$H/N_H = \frac{1}{k} \cdot \frac{S - G}{\sqrt{S + G}} \tag{5}$$

and this is the value to be optimized.

We used the optimization criterion 5 for two filters of emitted light. One of them was an interference filter with maximum transmittance at 400 nm and a bandpass of *ca.* 20 nm. This could supress the background fluorescence to a level below the sensitivity of the instrument. The second filter was a 400-nm cut-off filter that allowed for relatively high background fluorescence. With these two filters, we tested three mobile phases, and the results are given in Table I.

Table I shows that with an interference filter the optimization variable H/N_H is lower, even though the background is effectively zero. Better results were achieved with the cut-off filter. The results in Table I indicate that the use of a cut-off filter would improve the sensitivity by a factor of at least 5. The experimentally determined sensitivity enhancement under the conditions of real chromatography was about 100, as shown in Table II.

A calibration graph was established using plasma samples spiked with labetalol at levels ranging from 10–350 ng/ml, while the plasma internal standard concentration was kept at 2.5 μg/ml. The calibration parameters were calculated by the stan-

TABLE I

OPTIMIZATION OF FLUORESCENCE DETECTION

S = Sample signal; G = background mobile phase fluorescence; H/N_H = value calculated according to eqn. 5.

Mobile phase	*pH*	*Filter*					
		400 nm			*400 nm cut-off*		
		S	*G*	H/N_H	*S*	*G*	H/N_H
Citrate	6.5	18	0	4.2	448	47	18
Oxalate	7.0	20	0	4.5	485	17	21
Tartrate	7.0	24	0	4.9	574	58	20

TABLE II

H/N_H RATIO AND PEAK HEIGHTS OF LABETALOL WITH DIFFERENT FILTERS UNDER LC CONDITIONS

Concentration of labetalol, 500 ng/ml.

Parameter	*Emission filter*	
	400 nm	*400 nm cut-off*
H/N_H	4.2	18.0
Peak height (mm)	22.0	2024.0

TABLE III

INTRA-DAY ACCURACY OF LABETALOL DETERMINATION

Concentrations calculated as means of 10 measurements..

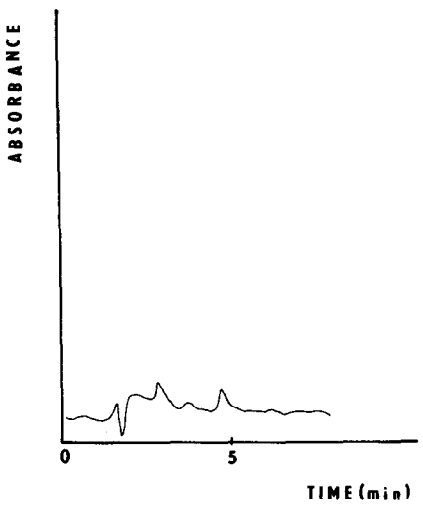
Theoretical concentration (ng/ml)	*Determined concentration (ng/ml)*	*Standard deviation (mg/ml)*	*Coefficient of variation (%)*
100.0	100.7	4.7	4.7
500.0	509.1	22.4	4.4

TABLE IV

INTER-DAY ACCURACY OF LABETALOL DETERMINATION

Concentrations calculated as means of 10 measurements.

Theoretical concentration (ng/ml)	*Determined concentration (ng/ml)*	*Standard deviation (ng/ml)*	*Coefficient of variation (%)*
100.0	100.5	4.2	4.2
1000.0	995.2	33.5	3.4



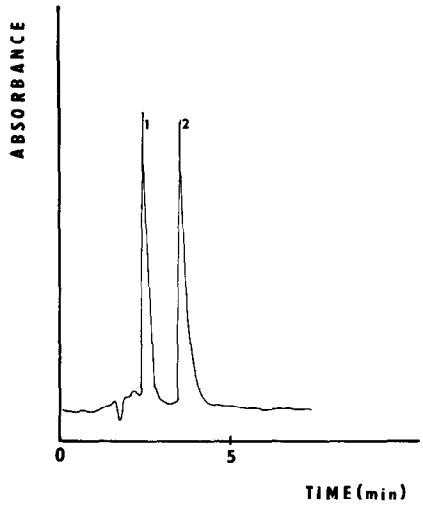


Fig. 2. Chromatogram of an extract from drug-free human plasma.

Fig. 3. Chromatogram of an extract from human plasma spike with 100 ng/ml of labetalol (1) and 2.5 μg/ml of internal standard (2).

dard linear regression procedure. The correlation coefficient of the measured calibration curve was 0.991. The intra-day accuracy was evaluated with plasma samples spiked at two known concentrations of labetalol. The results of ten determinations are given in Table III. The inter-day accuracy was determined by the analysis of ten replicate plasma samples spiked at two known concentrations of labetalol. The results (Table IV) show a very good reproducibility of the proposed method.

The recovery of labetalol by the extraction procedure was 79.5% and that of the internal standard was 81.3%. Even though the lowest concentration on the calibration graph was 10 ng/ml of labetalol in plasma, the signal-to-noise ratio (S/N) allowed a detection limit of 1 ng/ml (S/N = 3). No interference from plasma constituents was observed, as shown in the Figs. 2 and 3.

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