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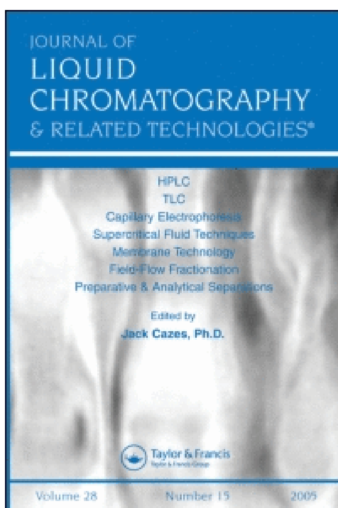

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

β-Blocking Agents: Determination of Biological Levels Using High Performance Liquid Chromatography

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To cite this Article Lefebvre, M. A. , Girault, J. and Fourtillan, J. B.(1981) 'β-Blocking Agents: Determination of Biological Levels Using High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 4: 3, 483 — 500
To link to this Article: DOI: 10.1080/01483918108059948
URL: http://dx.doi.org/10.1080/01483918108059948

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(3), 483-500 (1981)

β-BLOCKING AGENTS : DETERMINATION OF BIOLOGICAL LEVELS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Nine β-blocking agents have been tested and dosed by high performance liquid chromatography. Six of them, acebutolol an acebutolol metabolite, atenolol, metoprolol, propranolol and sotalol are detected with a fluorometric detector. Oxprenolol, pindolol and timolol can be quantified by their UV absorption at variable wavelength. A method is developped to find the best conditions of extraction and detection for each blocking agent. Experimental trials have led to a simple procedure for all compounds. Only pindolol and timolol plasma levels are non suitable for high performance liquid chromatography and need mass fragmentography or gas chromatography with electron capture detection.

However, pharmacokinetic parameters can be reached, for timolol and pindolol, through urinary excretion since sensitivity of the procedure is within the range of urinary levels.

The method has been applied, as well, to pharmacokinetic studies on sotalol, acebutolol, acebutolol metabolite, atenolol, propranolol, pindolol and timolol.

INTRODUCTION

Clinical studies have demonstrated large variations in β-blocking drug therapy. Two explanations can account for them. First, metabolism has been implicated for acebutolol and propranolol, and second, intestinal reabsorption after biliary excretion has been postulated. Since treatment by β-blocking agents for hypertension and angor usually involves long-term therapy, it would be of

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great interest to know plasma levels which can lead us to therapeutic schedules.

In this paper, we describe results of trials performed in an attempt to find conditions for plasma level determination of seven out of nine β-blocking drugs and urinary levels for all of them. In this study, sotalol plasma concentrations were the first studied by high performance liquid chromatography ; the procedure has been extended to other compounds.

This study included five steps :

- determination of wavelength in UV absorbance, excitation and emission wavelength in fluorometric detection for each blocking agent.
- determination of biological fluids alkalinisation conditions, since β-blocking agents are weak bases extractable in organic phase only as non-charged molecules.
- determination of the best extraction solvent among twelve commonly used organic solvents.
- determination of the best aqueous phase for back extraction, from organic layer.
- shaking times have been investigated and adaptation of mobile phase for high performance liquid chromatography has allowed good separation of drug, internal standards and endogenous components.

A large number of other cardiovascular drugs have been tested in order to determine eventual chromatographic interferences.

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MATERIALS AND METHODS

Equipment : The liquid chromatograph consisted of the following components : model 6000 A pump (Waters Associates, Paris, France), model WISP-710 A as automatic injector (Waters Associates, Paris, France) a μ-Bondapak C_{18} (Waters Associates, Paris, France). Three detectors have been used for these trials : model M 440 U.V. detector with 280 nm filter, M 450 U.V. variable wavelength and model 970 FS Schoeffel fluorometric detector (Cunow, Paris, France). The outputs of these detectors were connected either to a Houston Instruments chart recorder (Waters Associates, Paris, France) or to a Servotrace 10 mV (Sefram, Paris, France).

Flow rate was set for this entire study at 1.3 ml/min. for a 3100 psi pressure. To determine U.V. absorbance, a Pye Unicam

407 (Philips, Paris, France) instrument was used and a SPF 500 Aminco fluorometer (Kontron, Paris, France) allowed us to determine excitation and emission wavelengths for sotalol, acebutolol and its metabolite, atenolol, metoprolol and propranolol.

Reagents : U.V. grade acetonitrile and methanol were purchased from Prolabo (Paris, France). All organic solvents were reagent grade, obtained from Mallinckrodt, used without further purification. Hydrochloric acid, sodium hydroxide, acetic acid, sulfuric acid were Suprapur reagents from Merck. Borate buffer was prepared with doubly-distilled water.

In order to allow separation of weak bases such as blocking agents, counter ion 1-heptane sulfonic acid (PIC B-7 reagent, Waters Associates, Paris, France) was used and prepared by diluting one bottle of the reagent with one liter of mobile phase.

Other chemical products such as sodium sulfate were reagent grade quality.

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Method

- Conditions of detection : For the nine β-blocking agents, fluorometric spectra of excitation and emission were recorded with the Aminco fluorometer. These controls allowed us to set excitation wavelengths on Schoeffel detector, close to the maximum sensitivity, and eventually, to choose the emission filter. In the case of UV detection, UV absorption spectrum was measured with a Pye Unicam 407 ; Waters M 440 model with 280 nm filter or M 450 model with variable wavelength were used for chromatographic detection.

- Determination of plasma alkalinisation conditions : All blocking agents need to be extracted at basic pH with an organic solvent (Figure 3). Extraction were carried out from each β-blocking reference aqueous phase (1 ml), with 100 µl of 0.1 N, 0.5 N, N, 2 N, 4 N sodium hydroxide. After extraction and centrifugation, aliquots of aqueous phase were injected in the chromatograph. Peak heights were then compared with the β-blocking reference peak. For sotalol other trials were performed with aqueous phase set at pH 9, 9.2 and 10.

- Selection of best back extraction phase : Following first extraction by chloroform-n pentanol mixture, and back extraction with 0.01 N, 0.05 N, 0.1 N, 0.5 N, 1 N hydrochloric acid, 0.01 N, 0.05 N,

0.1 N sulfuric acid, 0.01 N, 0.05 N, 0.1 N, 1 N acetic acid, aqueous phase samples were injected into the chromatograph. Peak heights obtained from injection of aliqots of these aqueous phases were then compared with initial aqueous dilution of the drug. In the results we took into account the concentration obtained in back extraction aqueous phase since the volume of the later is three times smaller than the volume of the first aqueous phase.

- Other parameters : Extraction percentage with chloroform-pentanol 1 was measured following various time of agitation. Different mobile phases were tested in order to obtain the best separation of drug, internal standard and endogenous components from plasma.

For drug monitoring studies we tried a large number of drugs which could serve as internal standards for each β-blocking agent.

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RESULTS

- Conditions of detection : with UV absorbance, experience shows that M 440 with 280 nm filter was better for oxprenolol and pindolol and that M 450 was necessary for timolol, with wavelength set at 295 nm. For fluorometric detection, as shown in Figure 1 and 2, maximum of sensitivity was obtained with excitation wavelength set at 235 nm for sotalol and acebutolol, 222 nm for atenolol and 215 nm for propranolol. For acebutolol we used 389 nm filter while no filter was used for other blocking agents.

- Determination of plasma alkalinisation conditions : Except for sotalol, which was well extracted at pH 9, other blocking agents were extracted with best efficiency when 100 µl of 1 N NaOH was added to 1 ml of plasma (Table I).

- Determination of best extraction organic solvents : Chloroform-n-pentanol mixture seems to be the best extractant for the nine blocking agents (Table II). Dichloromethane-n-butanol resulted in emulsification and, therefore, was not used.

- Determination of best extraction phase : For all β-blocking agents, 0.1 N sulfuric acid gave excellent back extraction efficiency (Table III).

- Other parameters : Manually shaking was better than automatic shaking and two minutes were sufficient shaking time to provide excellent extraction.

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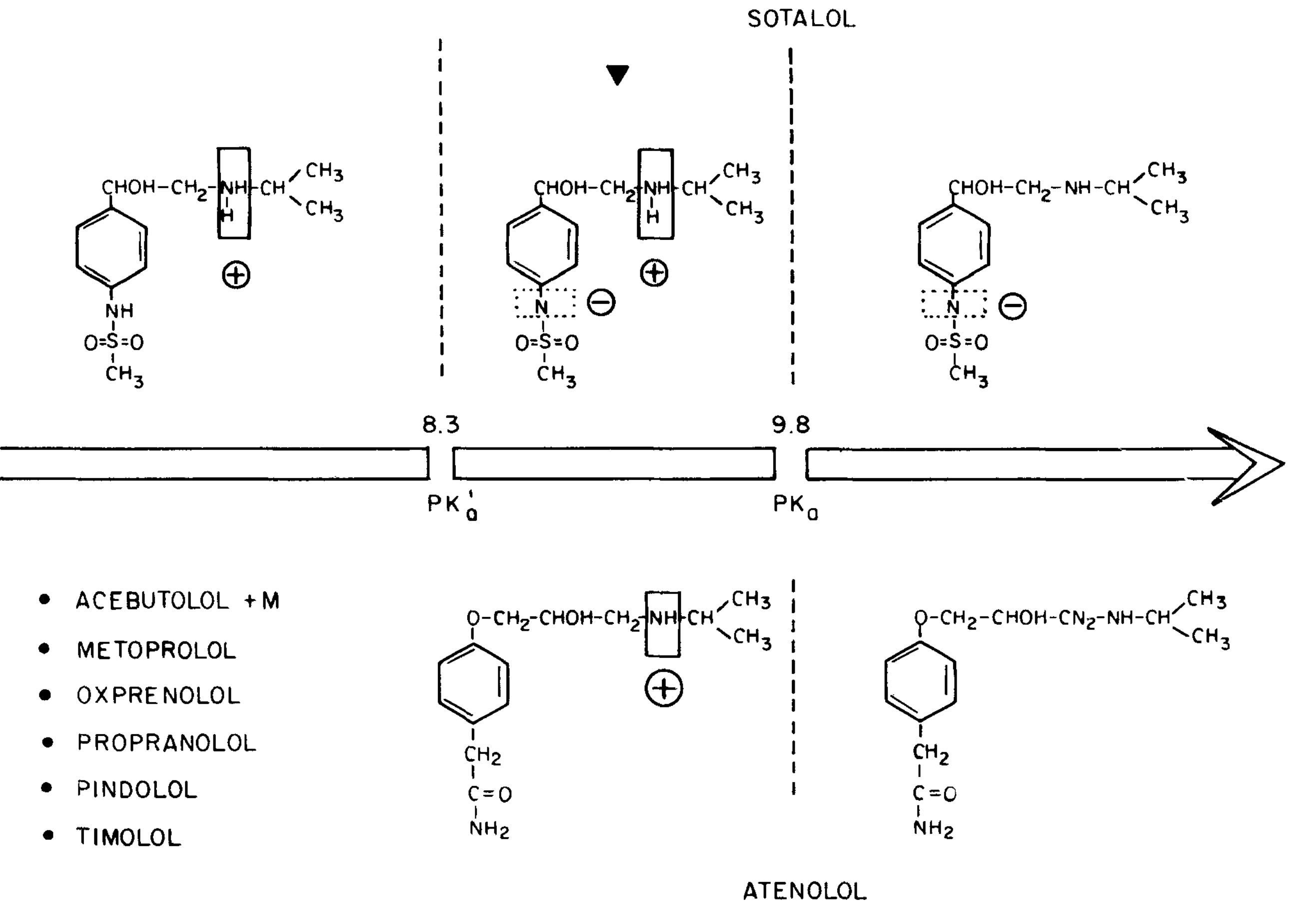

Fig. 1 - Ionisation state of blocking agents in relation with pH

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Tab. I - Influence of alkalinisation for plasma an extraction efficiency.
Except for sotalol,0.I ml of each basic solutions were added
to 1 ml of plasma.

	pH 9	0.1 N NaOH	0.5 N NaOH	N NaOH	2 N NaOH	4 N NaOH
Sotalol	72.6	pH 9.2 : 70	pH 10 : 37.9	NaOH 0.01 N : 19	0	0
Acebutolol		100	100	100	100	100
N Acébutolol		99	99	99	99	99
Atenolol		94.7	93.3	92.8	92.8	92.3
Metoprolol		99	99	99	99	99
Oxprenolol		≃ 100	≃ 100	≃ 100	≃ 100	≃ 100
Pindolol		≃ 100	≃ 100	≃ 100	≃ 100	≃ 100
Propranolol		≃ 100	≃ 100	≃ 100	≃ 100	≃ 100
Timolol		≃ 100	≃ 100	≃ 100	≃ 100	≃ 100

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Tab. II - Choice of best organic solvent of extraction

	Diethyl ether	Chloroform	Chloroform Pentanol 1 60-20 v/v	Dichlormethan	Dichlormethan Butanol 1 80-20 v/v	N-amyl acétate	Benzène	Hexan	Heptan	Toluène	Ethyl acétate	Cyclo hexan
Sotalol	10.2 %	23.6 %	72.6 %	24.8 %	75.2 %	22.3 %	3.8 %	3.2 %	4.5 %	7.0 %	57.9 %	5.7 %
Acebutolol	77.6	98.7	99.6	98.2	99.1	80.1	28.6	2.2	11.2	11.1	54.6	23.8
Acebutolol métabolite	33.2	86.0	99.1	82.6	99.1	85.5	24.9	20.5	20.8	22.6	62.6	19.1
Aténolol	44.7	77.0	96.9	75.2	95.1	56.5	47.8	48.4	49.1	50.3	40.9	43.5
Métoprolol	97.2	99.9	99.5	99.9	99.9	99.5	99.5	73.4	73.8	99.5	97.7	76.6
Oxprénolol	99.3	99.9	99.9	99.9	99.9	99.9	99.9	90.1	88.8	97.4	96.7	94.7
Pindolol	99.0	99.9	99.9	99.9	99.9	97.4	94.8	12.6	11.6	93.2	89.5	19.5
Propranolol	99.1	99.9	99.9	99.9	99.9	99.5	99.5	98.1	98.6	100	99.1	98.6
Timolol	99.9	99.9	99.9	99.9	99.9	67.7	99.9	73.2	72.9	99.9	99.9	99.9

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Tab. III - Choice of best back extraction aqueous phase

	HCl					H_2SO_4			CH_3COOH			
	0.01 N	0.05 N	0.1 N	0.5 N	N	0.01 N	0.05 N	0.1 N	0.01 N	0.05 N	0.1 N	1 N
SOTALOL	83.4	85.9	82.5	84.1	83.8	85.1	87.7	88.2	83.8	85	78.1	82.5
ACEBUTOLOL	38.5	36.3	35.1	15.0	10.5	54.8	70.5	80.5	5.2	32.0	43.7	57.4
M-ACEBUTOLOL	72.1	70.7	72.6	55.7	48.7	76.5	77.1	79.2	32.9	51.5	75.0	78.9
ATENOLOL	70.1	65.0	68.1	64.1	57.1	70.1	91.7	87.3	64.4	60.5	65.6	68.2
METOPROLOL	32.1	18.0	12.8	4.6	2.9	40.1	38.7	38.6	31.7	31.9	35.4	32.9
OXPRENOLOL	24.7	10.7	5.8	1.5	0.1	46.6	45.8	42.8	36.2	38.4	39.2	31.4
PINDOLOL	61.5	55	43.2	18.8	12.2	63.3	65	68.5	63.3	64.2	60.6	65
PROPRANOLOL	12.8	3.1	1.9	0.2	0.1	71.7	80.5	84.5	10.2	10.5	11.1	8.3
TIMOLOL	36.3	16.8	11.4	4.5	3.1	52.5	51.8	50	44.3	47.2	48.9	44.3

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Table 4 - Summary of results for nine blocking agents

	Detection Mode	Internal Standard (amount)	Retention time: Agent, Internal Standard (mn)	Limit of detection	Mobile phase Méthanol Acetic Acid water	Regression line	Coefficient correlation
Sotalol	(F) 235 nm-No Filter	Procaïnamide (10 µg)	(5.2 ; 7)	10 ng/ml	35 - 1 - 64	y = 1.19 x + 0.01	r = 0.998
Acebutolol	(F) 235 nm- F 389	LM 5008 (1 µg)	(3.6 ; 8)	5 ng/ml	50 - 1 - 49	y = 0.31 x + 0.01	r = 0.997
Acebutolol M	(F) 235 nm- F 389	LM 5008 (1 µg)	(6 ; 8.)	5 ng/ml	50 - 1 - 49	y = 0.515x + 0.08	r = 0.988
Atenolol	(F) 222 nm-No Filter	Procaïnamide (10 µg)	(6. ; 8.4)	10 ng/ml	28 - 1 - 71	y = 0.73 x + 0.04	r = 0.998
Métoprolol	UV 280 nm M 440	AcebutololM *(1 µg)	(4.8 ; 8.3)	10 ng/ml	50 - 1 - 49	y = 1.66 x + 0.06	r = 0.999
Oxprenolol	UV 280 nm M 440	PL 333 (20 µg)	(7.0 ; 9.8)	20 ng/ml	50 - 1 - 49	y = 0.306x + 0.01	r = 0.996
Pindolol	UV 280 nm M 440	Oxprenolol (10 µg)	(4.2 ; 6.6)	20 ng/ml	50 - 1 - 49	y = 1.57 x + 0.06	r = 0.988
Propranolol	(F) 215 nm-No Filter	LM 5008 (1 µg)	(8 ; 5.2)	0.50ng/ml	50 - 1 - 49	y = 2.95 x + 0.01	r = 0.996
Timolol	UV 295 nm M 450	PL 333 (160 µg)	(4 ; 6.4)	40 ng/ml	50 - 1 - 49	y = 0.64 x + 0.09	r = 0.997

We selected mobile phases with methanol acetic acid and water. Addition of counter-ion 1-heptane sulfonic acid to mobile phase is necessary for this kind of study.

Table 4 shows that mobile phases with 28 % (atenolol) 35 % (sotalol) and 50 % (others) were able to chromatograph the nine blocking agents. All these results have led us to develop the following standard extraction procedure.

- Standard extraction procedure : For both sotalol, acebutolol, acebutolol metabolite, atenolol, metoprolol, oxprenolol, pindolol, propranolol and timolol we used the following procedure : 0.9 ml of plasma and 0.1 ml of internal standard aqueous solution were added with 0.1 ml of 1 N sodium hydroxide and 7 ml of chloroform-n-pentanol mixture (60/20,v/v) in a 10 ml teflon-lined screw-capped tube. Tubes were shaken for two minutes and then centrifuged at 3500 rpm for 5 minutes. Aqueous phase was discarded and 6 ml of organic phase were placed with 300 µl of 0.1 sulfuric acid in new tubes which were shaking for two minutes and spun at 3500 rpm for five minutes.

Ten to 100 µl aqueous phase volume were taken for direct injection by an automatic injector (WISP-710), Waters Associates, Paris, France) into the H.P.L.C. system.

For propranolol plasma levels in order to prevent degradation of 4 Hydroxy-propranolol, sodium bisulfite (20 mg) was added before extraction.

For sotalol plasma levels, 0.33 ml of 2 N perchloric acid was added to 1 ml of plasma. Tubes were cooled for 10 minutes at 4°C and spun at 3500 rpm for 5 minutes. 1 ml of supernatant was then transferred with 100 1 of 4 N sodium hydroxyde and 2 ml borate buffer pH 9 in 10 ml teflon lined screw capped tubes. Seven ml of chloroform-n-pentanol (60/20, v/v) were then added and samples were treated as described above.

- Standard curves : Plasma samples were spiked with increasing amounts of each blocking agent and treated in the same manner as described above. Blocking agent concentrations for the trials were 4, 2, 1, 0.5, 0.25, 0.125, 0 µg/ml, respectively and internal standard concentration was variable. For propranolol the range was 0.25 - 0.0156 µg/ml. Each sample was performed in triplicate. Standard curves were then constructed by plotting the peak height ratios versus the drug concentration.

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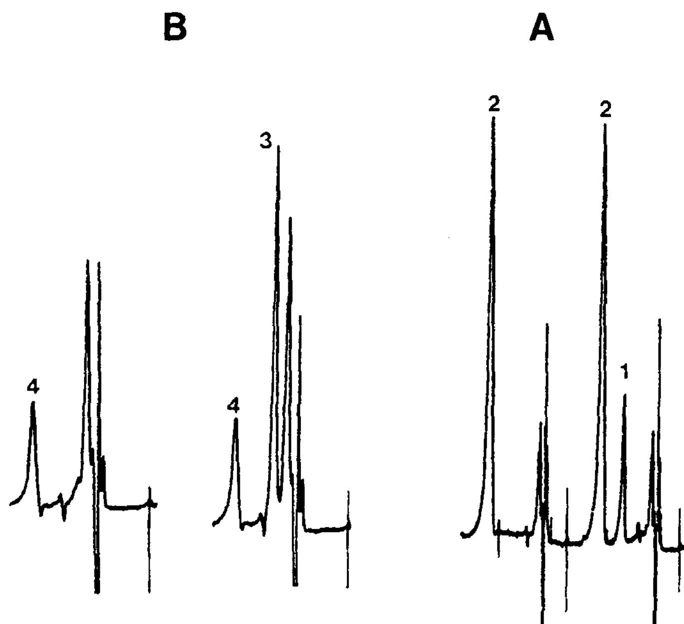

Figure 2.- Chromatograms of (A) plasma samples spiked with 1 μg/ml of Oxprenolol (1) and 20 mcg of PL 333 (2) - (B) Plasma sample spiked with 2 μg/ml of pindolol (3) and 10 mcg of oxprenolol (4). UV wavelengh 280 nm-mobile phase : methanol - acetic acid-water (50/1/49 - v/v/v)

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- U.V. detection : Figures 2, 3, 4, & 5 show typical chromatograms from plasma samples spiked with each blocking agent and its internal standard.

For pindolol, oxprenolol and timolol, mobile phase was methanol-water-acetic acid (50/49/1, v/v/v). Internal standard was Pl 333 (diphenyl 1,2 butyl-4 [N carbonyl oxypiperazinyl- N (hydroxy éthyl-2)] 5 pyrazoline 4, one 3) (obtained from LFT France) for oxprenolol and timolol, and oxprenolol for pindolol. No interference with endogenous components has been found.

The limits of sensitivity were about 20 ng/ml for pindolol and oxprenolol and 40 ng/ml for timolol. For oxprenolol pharmacokinetic studies, 12 H. plasma levels could be measured by taking 2 or 3 ml aliquots of plasma.

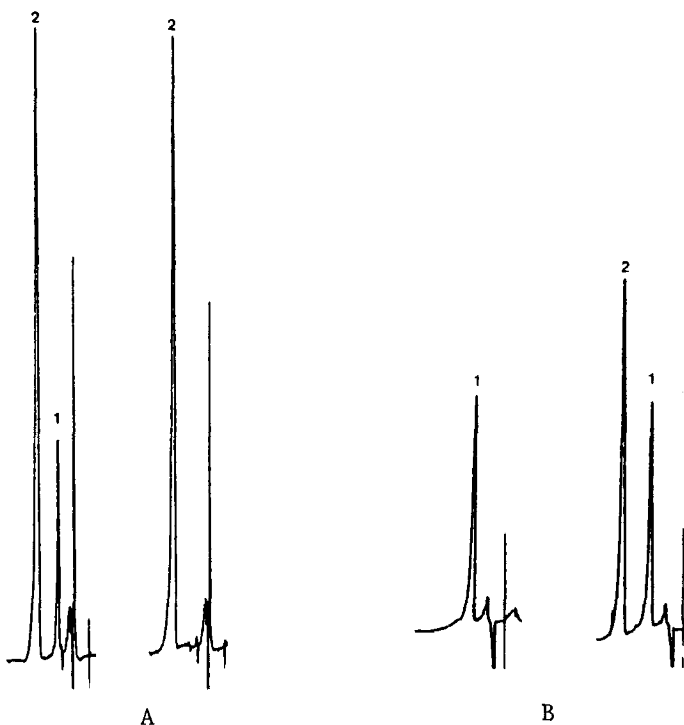

Figure 3 : A - Chromatogram of plasma samples spiked with 1 μg/ml of Timolol (1) and 160 μg of PL 333 (2) UV wavelength 295 nm. Mobile phase methanol-acetic acid-water (50/1/49 v/v/v).

B - Chromatogram of plasma samples spiked with 1 μg of metoprolol (2) and 1 μg of Acebutolol metabolite (1) excitation wavelength: 222 nm mobile phase (50/1/49 v/v/v).

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Timolol and pindolol plasma levels cannot be approached by high performance liquid chromatography, since 12 or 24 hours plasma levels are less than 1 ng/ml, but urinary levels, which can provide pharmacokinetic parameters, can be measured this way. Figure 6 depicts a linear relationship between the drug plasma levels and the peak height ratios for oxprenolol. Correlation

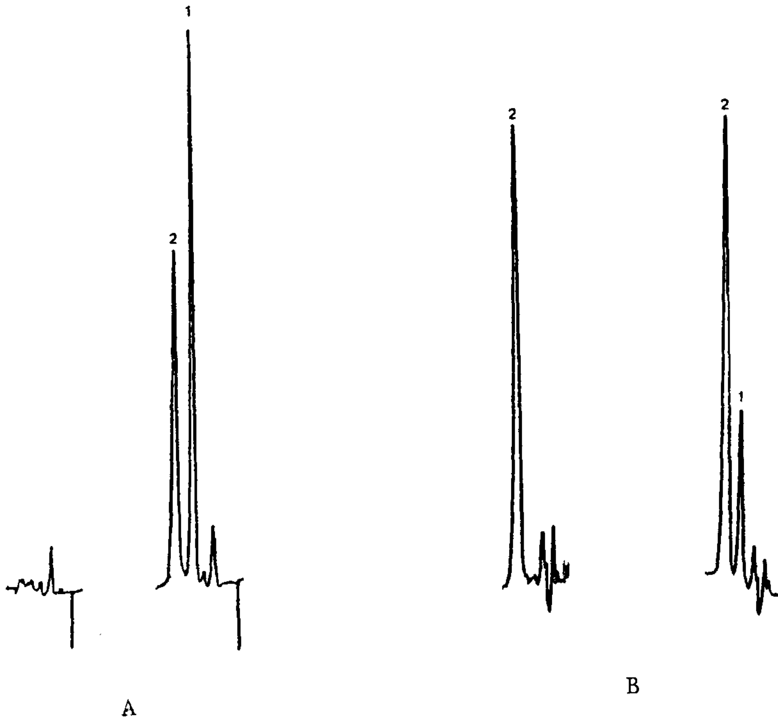

Figure 4 : A - Chromatogram of plasma samples spiked with 2 μg/ml of Sotalol (1) and 10 μg of procainamide (2) excitation wavelength : 235 nm. Mobile phase : meOH - acetic acid - water (35/1/64 v/v).
B - Chromatogram of plasma samples spiked with 0,5 μg/ml of Atenolol (1) and 10 μg of procainamide (2) excitation wavelength 222 nm. Mobile phase MeOH - Acetic acid - Water (28/1/71 v/v/v).

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coefficient was 0.997 for timolol. It was 0.988 and 0.996 for pindolol and oxprenolol respectively.

Fluorometric detection

For sotalol, atenolol, metoprolol and propranolol, for which excitation wavelengths were 235, 227, 222 and 215 nm, respectively, no filter was used at emission. For acebutolol a 389 nm cut

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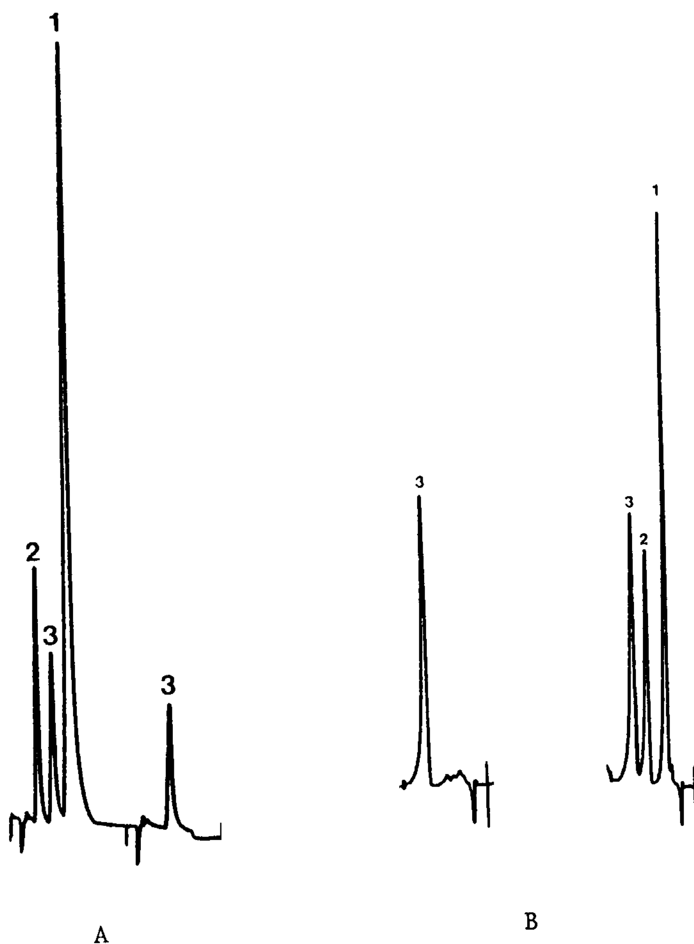

Figure 5 : A - Chromatograms of plasma samples spiked with 0.25 µg of Propranolol (1), 0,25 µg of OH Propranolol (2) and 1 g of LM 5008 (3). Excitation wavelength : 215 nm. Mobile phase : meOH acid water 50/1/49.

B - Chromatogram of plasma samples spiked with 2 µg/ml of Acebutolol metabolite (1) 2 µg/ml of Acebutolol (2) and 1 µg of LM 5008 (Internal Standard (3) excitation wavelength 235 nm (emission filter 389) Mobile phase : MeOH - Acetic acid - Water (50/1/49, v/v/v).

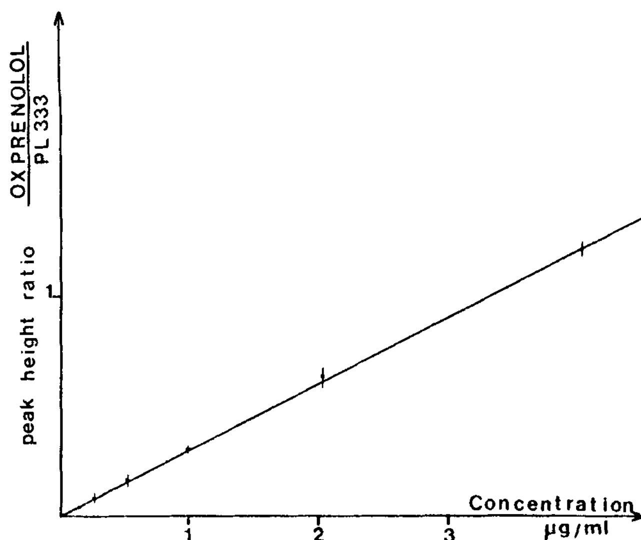

Figure 6 : Standard curve of Oxprenolol with Pl. 333 as internal satandard (range 4 - 0.125 µg/ml)

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off filter was used at emission with excitation wavelength set at 235 nm.

No interference with endogenous components from plasma has been found with fluorometric detection. Limits of sensitivity were 10 ng/ml for sotalol, atenolol and metoprolol, 5 ng/ml for acebutolol and its metabolite, and 0.5 ng/ml for propranolol. Internal standards were procainamide for sotalol and atenolol, and LM 5008 (4- [2-(3-indolyl) ethyl] piperidine) (Pharmuka, France) for acebutolol and propranolol.

DISCUSSION

The method previously described here allows measurement of plasma and urinary levels of β-blocking agents. We tried to develop a simple procedure common to all β-blocking agents. A great number of publications (1 to 18) are related to this category of drug. Most of them involve gas-liquid chromatography techniques which require derivatisation prior to injection. This step is very difficult to achieve, since temperature and time of derivatisation are critical.

High performance liquid chromatography does not deed such derivatisation and allows reduced analysis time compared to gas liquid chromatography. On the other hand, automatic injectors such as WISP 710 provide gain of time for pharmacokinetic studies.

The twostep extraction procedure first leads to a sample clean-up of plasma, and second, allows direct injection into the chromatograph of the aqueous phase from back extraction. Moreover, concentration of the drug is achieved since the aqueous phase has a volume three time lower than that of the initial plasma sample.

Three detector models were used for these studies. U.V. absorbance detector M-440 with 280 nm filter provides an excellent baseline for chromatograms and allows increased sensitivity, since 20 ng of oxprenolol and pindolol per milliliter of plasma can be quantified with excellent precision and accuracy. For timolol plasma levels, we used M 450 variable wavelength detector set at 295 nm. The sensitivity is about 40 ng/ml with this detector.

For other β-blocking agents, fluorometric detector FS 970 (Schoeffel) was used successfully ; excitation wavelength set between 215 and 235 nm gave, with our extraction procedure, no interference between drugs and endogenous components from plasma. Limits of sensitivity for atenolol, sotalol, propranolol, metoprolol, acebutolol and its metabolite are suitable with pharmacokinetic studies.

Retention times of all compounds are less than 10 minutes and total analysis time never exceeds 45 minutes, since conditioning of the column could be reached within 30 minutes and extraction procedure for one blocking agent with its internal standard takes about 30 minutes.

On the other hand, only one organic solvent (Chloroform-n-pentalol (60/20, v/v) and one back extraction aqueous phase are required for the procedure. Internal aqueous dilutions are prepared monthly and kept at 4°C without degradation.

Considering the mobile phase, good chromatographic separations were performed with methanol percentage adjusted to 28 % for atenolol, 35 % for sotalol and acebutolol, and 50 % for other β-blocking drugs. In all cases, addition of 1-heptane sulfonic acid was necessary.

Finally, for drug monitoring studies as well as for pharmacokinetic studies, the procedure is simple and provides a

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reduced analysis time. It could be applied for a great number of pharmaceutical compounds from which structures and ionisation states are similar to β-blocking agents.

AKNOWLEDGMENT

We would like to thank Mr. B. JULIAN and P. GOBIN for their technical assistance throughtout the work.

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