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Sensitive high-performance liquid chromatographic method for the determination of labetalol diastereoisomers in plasma samples without derivatization

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Abstract

A direct high-performance liquid chromatographic assay for the determination of labetalol diastereoisomers in plasma without derivatization was developed. Baseline resolution of diastereoisomers was accomplished on a C_{18} bonded reversed-phase polymeric column with a basic (pH 11.5) mobile phase and isocratic elution. Sample treatment was optimized in order to achieve a complete extraction of labetalol diastereoisomers and to avoid racemization during extraction. Fluorimetric detection improved the selectivity and afforded a detection limit of 3 ng/ml for each diastereoisomer. This method is suitable for routine quantification of labetalol diastereoisomers and has been applied to a pharmacokinetic study in small laboratory animals.

1. Introduction

Labetalol hydrochloride, 5-[1-hydroxy-2-((1-methyl-3-phenylpropyl)amino)ethyl]salicylamide monohydrochloride, is an effective antihypertensive agent with both α_1 and β_1 adrenoreceptor blocking activity [1]. Because the labetalol molecule contains two chiral centers, there are four possible stereoisomers (*RR*, *RS*, *SR*, *SS*) that can form two diastereomeric pairs of racemates (*RR-SS* and *RS-SR*). The pharmacological properties of the individual stereoisomers, however, differ considerably in that most of the α_1 adrenoreceptor blocking activity is due to the *SR* isomer, whereas most of the β_1 adrenoreceptor blocking activity is attributable to the *RR* isomer [2–4]. Furthermore, evidence has been presented by different authors [5,6] that labetalol may be subject to stereoselective metabolism and disposition.

In order to study the stereoselectivity of labetalol pharmacokinetics, to improve drug monitoring and to maintain an adequate control of commercially available products, a sensitive and reliable methodology which can separate the labetalol stereoisomers is required. Many methods are at present available to assay labetalol both in plasma and pharmaceutical products [7–26]. However, little has been published on the chromatographic separation of labetalol stereoisomers in biological fluids.

Schill *et al.* [19] and Lalonde *et al.* [6] reported a direct high-performance liquid chromatographic (HPLC) separation of the four stereoiso-

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mers of labetalol in a single run using an α_1-acid glycoprotein chiral column, but these authors did not provide sufficient information to determine the extend of separation achieved. Lalonde *et al.* [6] attempted to determine the four stereoisomers in human blood plasma but the assay was not sufficiently sensitive to permit a full pharmacokinetic study of the four isomers. More recently, Desai and Gal [20] described a reversed-phase HPLC methodology for the separation of the four stereoisomers of labetalol via derivatization with a chiral isothiocyanate reagent. However, this procedure was not applied to the analysis of the isomers in biological media.

Several reports have described the chromatographic separation of the diastereoisomers *RR-SS* and *RS-SR* of labetalol by gas chromatography (GC) or HPLC. Most of these methods are based on indirect techniques which are dependent on various derivatization procedures [21–25]. Munro *et al.* [21] reported an indirect GC separation of the diastereoisomers of labetalol as their cyclic boronate derivates which was further used by Goromaru *et al.* [22] for their quantification from urine. This boronate GC method was later improved and validated by Cholerton *et al.* [23] but its application was restricted to the determination of the "racemate ratio" in pharmaceutical products.

Selby and Munden [24] described the first HPLC separation of the labetalol diastereoisomers but noted difficulties to obtain reliable data from this procedure. Linder *et al.* [25] later reported an HPLC separation of labetalol isomers as their O,O-dibenzoyltartaric acid monoesters. However, they did not state whether the two diastereoisomers, or the enantiomers of a given racemate, had been separated. More recently, Sztruhar *et al.* [26] described a direct, isocratic, reversed-phase HPLC procedure for the separation of the diastereoisomers of labetalol and proposed the application to pharmaceutical analysis such as quality control.

At present, it is clear that the available chromatographic methods are not sufficiently sensitive to permit a full pharmacokinetic study of labetalol enantiomers or diastereoisomers. Thus in this paper we re-examined the direct HPLC method of Sztruhar *et al.* [26] which is simple and convenient to carry out in order to apply it to the quantification of the labetalol diastereoisomers in plasma samples.

2. Experimental

2.1. Apparatus

Analyses were performed on an HP 1050 liquid chromatographic system (Hewlett-Packard, Evry, France) consisting of a solvent delivery system, an automatic injector and an electronic integrator. A Kratos Spectroflow 980 fluorimeter (Kratos, Paris, France) was used as the detector with an excitation wavelength of 340 nm and an emission wavelength of 389 nm. The time constant was set at 2 s and the output signal ranged between 0.01 and 0.1 μA. The signal from the detector was connected to the integrator in order to generate real-time chromatographic tracings and to integrate peak areas.

2.2. Chemicals

Labetalol hydrochloride was obtained from Sigma (St. Quentin-Fallavier, France) as an approximately equimolar mixture of *RR-SS* and *RS-SR* diastereoisomers. Separation of labetalol into its diastereoisomers was easily carried out according to the technique published by Gold *et al.* [3]. This procedure involved fractional crystallization of labetalol by taking advantage of the extreme insolubility of the tosylate salt of the *RR-SS* diastereoisomer and the solubility of the acetate salt of the *RS-SR* diastereoisomers in 2-propanol.

All other chemicals were obtained from Merck (Darmstadt, Germany) and were of analytical grade, except for HPLC grade acetonitrile which was purchased from Rathburn chemicals (Infolabo, Ste Foy la Grande, France). The carbonate buffer (pH 8.8) contained 3.80 g/l of $NaHCO_3$ and 0.504 g/l of Na_2CO_3.

2.3. Chromatographic conditions

Reversed-phase separations were performed on a 150 × 6 mm I.D. stainless-steel column commercially packed (Prolabo, Paris, France) with octadecyl-bonded polymer gel of 5-μm particle diameter (Asahipack ODP 50 column).

The mobile phase consisted of 0.05 *M* diethylamine pH 11.5–acetonitrile (160:840, v/v) and in 0.036 *M* sodium chloride. It was filtered and degassed under reduced pressure daily before use. The system was operated at ambient temperature with a flow-rate of 0.9 ml/min.

2.4. Standard solution

A stock solution of labetalol hydrochloride (an approximately equimolar mixture of the diastereoisomers) was prepared in distilled water at a concentration of 100 μg/ml free base form. Subsequent dilutions with distilled water were such that the plasma standards could be prepared conveniently by adding a 100-μl volume to 900 μl blank plasma. Aqueous solutions of *RR-SS* and *RS-SR* diastereoisomers of labetalol were also prepared (approximate concentration 1 μg/ml) and injected to verify the chromatographic peaks of the diastereoisomers.

2.5. Treatment of plasma samples

An aliquot (0.5 ml) of plasma was transferred to a 15 ml glass tube fitted with a Polytef lined screw cap. The plasma sample was then diluted with 1 ml of carbonate buffer (pH 8.8) and extracted with 10 ml of a chloroform–isopropanol (100:2, v/v) mixture by agitation on an horizontal reciprocal shaker (Toulemonde, Paris, France) for 10 min (30 strokes per min). After centrifugation (1000 *g* during 10 min) the aqueous layer was discarded. An 8-ml volume of the organic phase was transferred into a conical tube and evaporated to dryness under a stream of nitrogen. The residue was then redissolved in 50 μl of the following mixture: acetonitrile–methanol–0.1 *M* HCl (30:20:5, v/v/v) and 25 μl of this solution was injected into the liquid chromatograph.

2.6. Calibration curves

The procedure of Gold *et al.* [3] separates the *RR-SS* and the *RS-SR* diastereoisomers of labetalol, but their degree of purity (70–75%) was not suitable for the preparation of standard plasma samples.

Standard samples for calibration were consequently prepared from the stock solutions of labetalol hydrochloride (mixture of diastereoisomers, 6.25–400 ng/ml). Concentrations of the two diastereoisomers in standard plasma samples were calculated according to the following formulas: C (of each diastereoisomer) = C labetalol · P; P = percentage of each diastereoisomer in the mixture (mean value of ten different chromatographic measurements of the stock solution of labetalol HCl). Calibration curves were obtained by linear regression of the peak areas of the *RR-SS* and the *RS-SR* diastereoisomers *versus* their concentrations in the standards.

2.7. Recovery, accuracy and precision

Inter- and intra-assay precision and accuracy were determined at three concentrations of labetalol hydrochloride (mixtures of diastereoisomers) (400, 100 and 10 ng/ml) (n = 8). Recovery was calculated by comparing the measured values of spiked samples with those of standard aqueous solutions at three concentrations (400, 100 and 10 ng/ml) of labetalol hydrochloride.

3. Results and discussion

3.1. Chromatography

Our results confirm the observation of Sztruhar *et al.* [26] that an adequate HPLC separation of the two diastereoisomers of labetalol may be obtained by direct means using a reversed-phase column and a basic mobile phase (see Fig. 1B). This separation is dramatically dependent on the mobile phase pH (Fig. 2), suggesting that the separation of the dia-

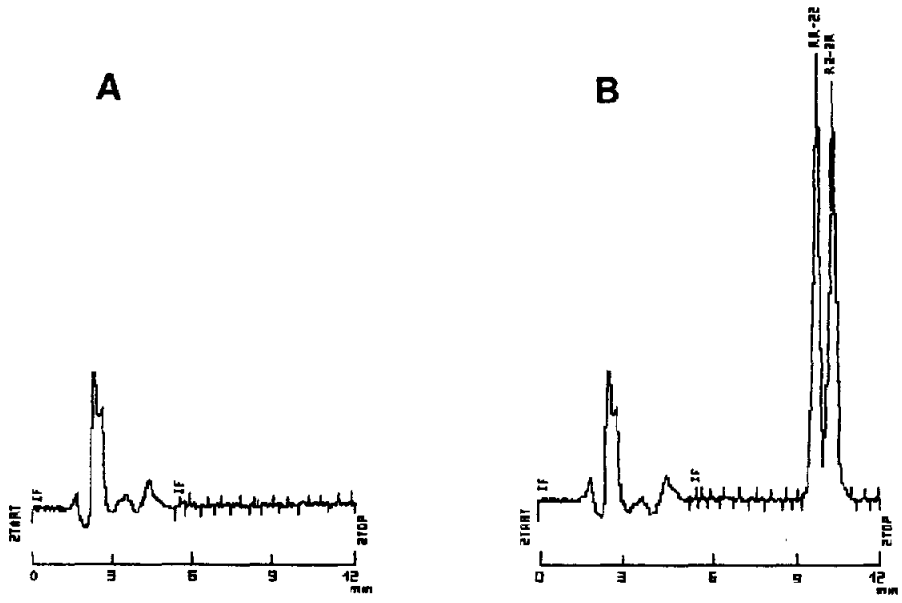

Fig. 1. Representative chromatograms of the diastereoisomers of labetalol in extracts of drug-free plasma (A) and labetalol containing plasma (B). (B) Sample from the pharmacokinetic study [rat 1, time of sampling: 1.9 h, measured concentrations: 62 ng/ml (*RR-SS*) and 65.05 ng/ml (*RS-SR*)].

stereoisomers is dependent on their degree of ionisation. No separation was found with an acidic mobile phase. Resolution occured at a pH higher than 10.5 reaching its maximum at pH 12. Thus, optimisation of the chromatographic separation of the labetalol diastereoisomers requires the use of a basic mobile phase with a pH higher than 12.

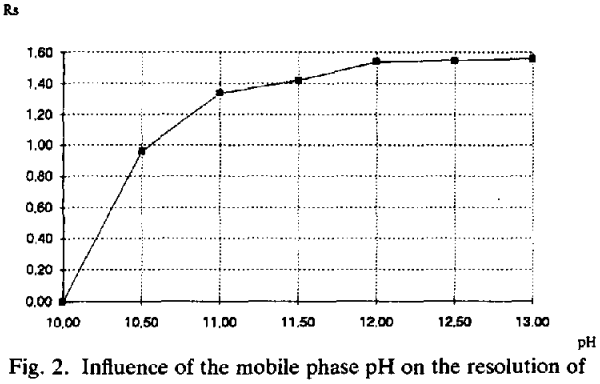

Fig. 2. Influence of the mobile phase pH on the resolution of labetalol hydrochloride diastereoisomers. Resolution factor (R_s) is computed as

$$R_s = \frac{2(tR_{(RS\text{-}SR)} - tR_{(RR\text{-}SS)})}{w_{(RS\text{-}SR)} + w_{(RR\text{-}SS)}}.$$

We also studied the influence of the mobile phase buffer on the resolution (R_s) and the retention (k') of the labetalol diastereoisomers. No significant differences were found for the resolution factors of the diastereoisomers at 4 concentrations of 3 different buffers (sodium borate–NaOH, glycine–NaOH, disodium phosphate–NaOH). In contrast, the concentration of the mobile phase buffer does effect the retention of the labetalol diastereoisomers. Increasing concentrations of disodium phosphate–NaOH buffer increased the capacity factor (Fig. 3). This might be due to the formation of ion-pairing with sodium.

In order to test this hypothesis, successive analyses have been performed using an alkaline mobile phase (pH 11.5) containing increasing concentrations of sodium chloride or disodium sulfate. The results (Fig. 4) demonstrate that the retention of the labetalol diastereoisomers depends on the sodium ion concentration. This confirms the ion-pair formation between Na^+ and probably the phenolic hydroxyl group of labetalol.

For the assay of the labetalol diastereoisomers

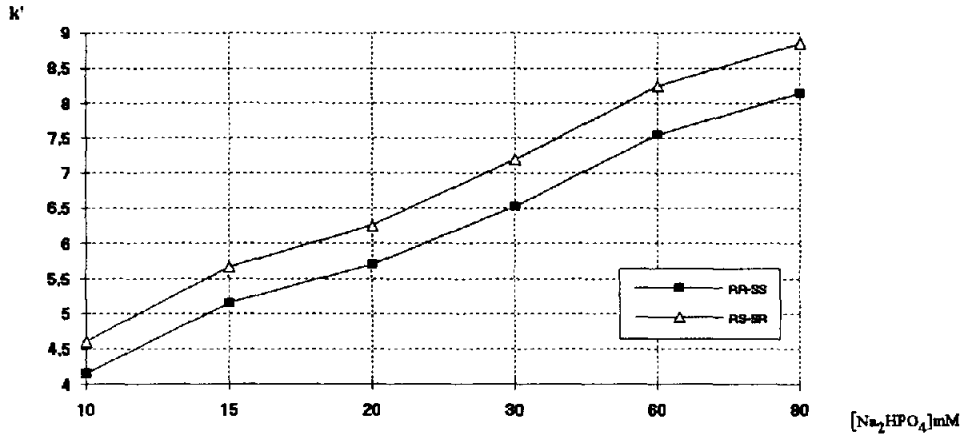

Fig. 3. Influence of increasing concentrations of disodium phosphate [Na_2HPO_4]/NaOH buffer on the capacity factor (k') of the labetalol diastereoisomers.

in plasma samples, chromatography was finally performed using an alkaline mobile phase (pH 11.5) containing diethylamine and 0.036 *M* NaCl. These chromatographic conditions are slightly different from those proposed by Sztruhar *et al.* [26]: a higher value for the mobile phase pH (11.5 *versus* 10.8), diethylamine instead of ammonia. The choice of pH 11.5 increases the separation of the labetalol diastereoisomers without, however, reaching its optimum. However this choice represents a good compromise between the fluorescence of labetalol, the stability of the Asahipack column and the resolution of the diastereoisomers. The addition of diethylamine alkalinizes the mobile phase and maintains its pH. Sodium chloride was added to retard the elution of corresponding diastereoisomer peaks in order to avoid interferences with endogenous peaks.

The chromatogram of a drug-free plasma is shown in Fig. 1A. A representative chromatogram of a plasma sample is shown in Fig. 1B. Previous HPLC work [6,10,13] employed a derivative of salicylamide and chloroquine as internal standard. These two drugs are fluorescent under alkaline conditions. Nevertheless, we did

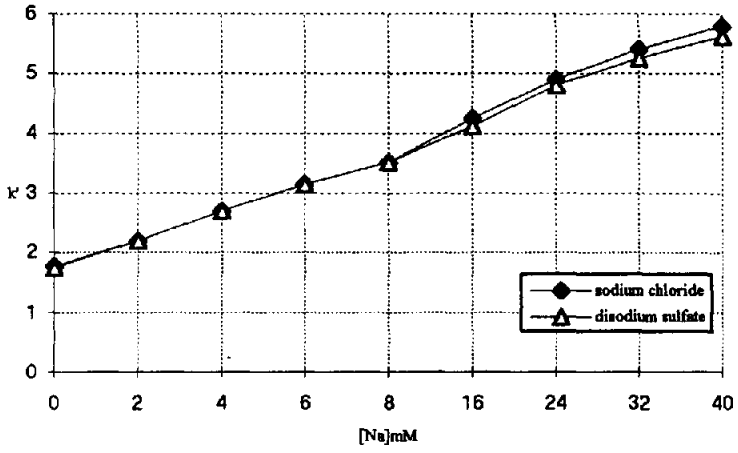

Fig. 4. Influence of sodium concentration [Na] on the capacity factor (k') of the diastereoisomers of labetalol. For each diastereoisomer, the variations of the capacity factor (k') are similar for the two tested salts (Na_2SO_4, NaCl) when k' is expressed as a function of sodium concentration.

not find these drugs to be satisfactory internal standards because they elute too late under the chromatographic conditions used here. We failed in this study to find a convenient internal standard, but its absence does not affect dramatically the precision and the accuracy of the assay.

All calibration curves were found to be linear for concentrations ranging between 0 and 500 ng/ml with correlation coefficients higher than 0.99. The precision and accuracy of replicate analyses of the same sample expressed as relative standard and percentage bias ranged from 1.9 to 14.9% (Table 1). Day-to-day reproducibility of the method was equivalent (Table 2) for the tested concentrations. In spite of the absence of an internal standard, the satisfactory precision and accuracy of the assay are probably due to the complete extraction of the labetalol diastereoisomers from plasma samples (Table 3).

The extraction of labetalol has also been

Table 1
Intra-day precision and accuracy of labetalol diastereoisomer assay in plasma samples

Theorical concentration (ng/ml)		Mean observed concentration (ng/ml)		Coefficient of variation (%)		Percentage bias, relative accuracy (%)	
RR-SS	*RS-SR*	*RR-SS*	*RS-SR*	*RR-SS*	*RS-SR*	*RR-SS*	*RS-SR*
196	204	214	223	4	4.4	+9.5	+9.6
49	51	49.4	50.05	8.5	8.2	+0.85	−1.9
4.9	5.1	4.55	4.65	14.9	4.4	−6.76	−9.63

Table 2
Inter-day precision and accuracy of labetalol diastereoisomer assay in plasma samples

Theorical concentration (ng/ml)		Mean observed concentration (ng/ml)		Coefficient of variation (%)		Percentage bias, relative accuracy (%)	
RR-SS	*RS-SR*	*RR-SS*	*RS-SR*	*RR-SS*	*RS-SR*	*RR-SS*	*RS-SR*
196	204	207	219	6.7	7.9	+5.6	+7.3
49	51	53	55	11	10.3	+8.1	+7.8
4.9	5.1	5.4	5.6	13	13.7	+10	+9.8

Table 3
Mean percentage recovery of labetalol diastereoisomers from plasma at varying concentrations

n	Mean labetalol diastereoisomers concentration (ng/ml)		Mean recovery of labetalol diastereoisomers (%)		Mean recovery of labetalol diastereoisomers calculated for a complete (10 ml) collection of organic phase (%)	
	RR-SS	*RS-SR*	*RR-SS*	*RS-SR*	*RR-SS*	*RS-SR*
8	196	204	80.8	81.6	101	102
8	49	51	77.6	79.8	97	96
8	4.9	5.1	76.8	76.0	96	95

investigated by other workers [6,8,10,13,27,28]. Verbesslt *et al.* [27] and Lalonde *et al.* [6] described a solid-phase extraction procedure for labetalol from plasma using an octadecylsilane extraction column. However these authors did not provide sufficient information about the extraction recoveries. Several authors [6,8,10,13] have investigated liquid–liquid extraction of labetalol and have demonstrated that complete extraction of labetalol may be achieved using a rather polar solvent and alkaline conditions: pH 8–9. Despite the formation of an emulsion with plasma at alkaline pH, chloroform was used in this work because of its ability to extract completely the labetalol diastereoisomers, to avoid racemisation during extraction, and to clean-up the samples.

The fluorimetric detection and the total extraction of labetalol diastereoisomers under the above conditions improve the selectivity and sensitivity of the method. Although we did not measure concentrations below 7 ng/ml, the signal-to-noise ratio indicates a detection limit down to 3 ng/ml.

3.2. Pharmacokinetic application

To evaluate this assay for pharmacokinetic studies, the disposition of labetalol diastereoisomers was examined in three male Sprague-Dawley rats (body weight 560 to 600 g). Under short ether anesthesia, catheters (Biotrol, France) were inserted into the left femoral and the right jugular veins and exteriorized subcutaneously through the back of the neck. After allowing the animals to recuperate overnight, a 1.5 mg/kg dose of labetalol hydrochloride (diastereoisomers mixture) was administered through the femoral catheter. Blood samples were collected via the jugular catheter at 5, 10, 30 min and 2, 4, 6 h.

Fig. 5 shows the mean plasma concentration–time curves for labetalol diastereoisomers for the 3 rats who received a 1.5 mg/kg intravenous bolus.

4. Conclusions

The chromatographic method described here was suitable to study the pharmacokinetic profiles of labetalol diastereoisomers since the concentrations remained higher than the validated lower limit of quantification throughout the 6-h period of plasma collection. Pharmacokinetic data were analysed by compartmental and non-compartmental methods. These methods yielded similar results (Table 4). Despite the limited information provided in this study, the observed difference in elimination half-lives of the 2 dia-

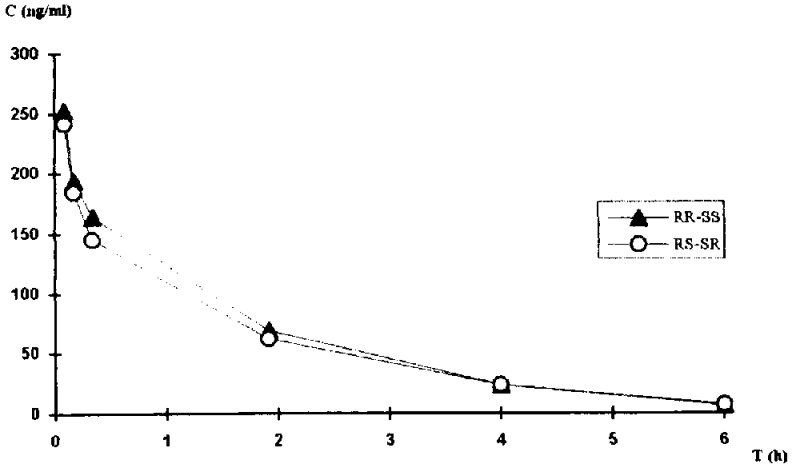

Fig. 5. Mean plasma concentration (C)–time (T) curves for labetalol diastereoisomers (*RR-SS* and *RS-SR*) for the 3 rats who received 1.5 mg/kg labetalol intravenous bolus.

Table 4
Mean pharmacokinetic parameters of labetalol diastereoisomers in rat after intravenous injection

Pharmacokinetic parameter	Mean value in rats after intravenous injection	
	RR-SS	*RS-SR*
Elimination half-life ($t_{1/2}\beta$) (h)	1.15	1.35
Total clearance (Cl_t) (l/h)	1.21	1.09
Volume of distribution (V_d) (l)	2.06	2.14

stereoisomers is consistent with a stereoselective disposition of labetalol. Work is now in progress to confirm these preliminary results and to elucidate the origin of the stereoselective disposition in rat.

5. References

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