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Sensitive high-performance liquid chromatographic method for direct separation of labetalol stereoisomers in biological fluids using an α_1-acid glycoprotein stationary phase

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ABSTRACT

A chiral high-performance liquid chromatographic assay for the separation of the four stereoisomers of labetalol, an antihypertensive, in biological fluids has been developed. Baseline separation of the isomers was achieved using an α_1-acid glycoprotein stationary phase. No interference from endogenous substances was observed following extraction from various biological fluids obtained from pregnant (ewe and fetus) and non-pregnant sheep. The concentration of the individual isomers of labetalol was determined by first measuring the total concentration of racemic labetalol obtained from an achiral assay followed by reassay of each sample by the chiral method after which, by using the estimate of the percentage of each individual isomer, the individual concentration of each of the four isomers was determined. The mobile phase was 0.02 *M* phosphate buffer containing 0.015 *M* tetrabutylammonium phosphate. The pH of the mobile phase was adjusted to 7.10. The detector was set at an excitation wavelength of 230 nm and emission wavelength of 400 nm to monitor the nascent fluorescence intensity of the isomers of labetalol. The limit of detection of the individual isomers was 0.15 ng (0.6 ng of injected racemic labetalol). The assay was linear over the range 0.6–15.0 ng of labetalol (injected) with the intra- and inter-day mean coefficients of variation being less than 9.0 and 6.0%, respectively. Application of the assay in the study of pharmacokinetics of the stereoisomers of labetalol in sheep following administration of racemic labetalol has been demonstrated.

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INTRODUCTION

Labetalol hydrochloride, 5-{1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl}salicylamide, is an α_1- and β-adrenoceptor antagonist with partial β_2-agonist activity [1,2]. The molecule contains two chiral centers resulting in four stereoisomers; *SR*, *SS*, *RS* and *RR*. Labetalol is marketed as an approximately equicomponent mixture of all four stereoisomers (Trandate) [3]. The pharmacological activities of the four stereoisomers have been shown to differ greatly from one another [4,5] but there is little information available on the stereoselective pharmacokinetics of this drug largely due to the lack of a suitable chiral assay. While attempts have been made in the past to separate the isomers of labetalol by HPLC [6–8], details of the methods and their applicability in pharmacokinetic studies were not adequately discussed. Schill *et al.* [6,7] demonstrated the potential of α_1-acid glycoprotein (AGP) as a stationary phase for the chiral separation of a number of racemic drugs, including labetalol A and B (the diastereomers of labetalol). Lalonde *et al.* [8] suggested stereoselectivity in the clinical pharmacokinetics of labetalol in nine healthy male volunteers who received either 1.2 mg/kg intravenously or 200 mg orally. However, the stereoselectivity was based on the proportion of the individual isomers in one sample point corresponding to the highest concentration. The details of the assay including the degree of resolution or a representative chromatogram were not provided. Recently, Desai and Gal [9] reported a method for the separation of labetalol isomers which involves derivatization with chiral [(4*S*-*cis*)2,2-dimethyl-5-isothiocyanato-4-phenyl-1,3-dioxane] and non-chiral [benzyl isothiocyanate and 1-naphthalenemethyl isothiocyanate] reagents. The primary objective of the current study was to develop a rapid but sensitive assay for the direct separation of the stereoisomers of labetalol in biological fluids. We report here a method for the baseline separation of labetalol stereoisomers with a minimum quantitation limit of 0.15 ng for each isomer (amount injected). Application of the assay in the study of stereospecific pharmacokinetics of labetalol in non-pregnant sheep is demonstrated.

EXPERIMENTAL

Materials

Labetalol hydrochloride was purchased from Sigma (St. Louis, MO, USA). The isomers of labetalol (*SR*, *SS*, *RS* and *RR*) and the internal standard, 5-{2-[4-(4-methylphenyl)-2-butylamino]-1-hydroxyethyl}salicylamidehydrochloride hemihydrate (SCH), were generously supplied by Schering Plough (Bloomfield, NJ, USA). Potassium dihydrogen orthophosphate and disodium hydrogen orthophosphate were purchased from BDH (Toronto, Canada). Reagent-grade phosphoric acid was purchased from Caledon Labs. (Georgetown, Canada). The cationic modifier tetrabutylammonium phosphate buffer, TBAP (0.5 *M*, pH 7.5), was obtained from Regis (Morton Grove, IL, USA). Ethyl acetate (HPLC grade 256) was purchased from Caledon Labs. HPLC-grade water was obtained through a Milli-Q water system (Millipore, Bedford, MA, USA).

The stock solutions of labetalol and each individual isomer were prepared by dissolving them in methanol. Subsequent dilutions of these solutions were made with dilute phosphoric acid.

Chromatography

An HP 1090 liquid chromatographic system (Hewlett Packard, Palo Alto, CA, USA) equipped with an autoinjector, autosampler, sample loop injector of 250 μl capacity and HP 79994A Pascal analytical workstation (Model 310) was used for analysis. Labetalol isomers were separated on a Chiral AGP column (10 cm × 4 mm I.D., 10 μm sorbent particle size) obtained from Regis. A 10 mm × 3.0 mm Chiral AGP guard column (Regis) was placed before the Chiral AGP analytical column.

A Model HP 1046A programmable fluorescence detector (Hewlett Packard) was used with the excitation wavelength set at 230 nm and emission wavelength at 400 nm to monitor the nascent fluorescence intensity of the isomers of labetalol. Other detector components included a 370-

nm emission cut-off filter, a 2-mm-wide excitation slit and two 4-mm-wide emission slits as described previously [1].

Animal experiments

Non-pregnant ewes of Suffolk, Finn and Dorset breeds were used in these experiments. The procedure used in the catheterization of sheep [10] and the protocol employed in the infusion of labetalol are described elsewhere [11]. Briefly, a 100-mg intravenous bolus of labetalol was followed immediately by an infusion at a rate of 0.5 mg/min for 6 h. Plasma samples were obtained at −15, 15, 30, 45, 60, 90 and 120 min, and every hour until 6 h during infusion and at 15, 30, 45, 60, 90 and 120 min, 3 and 4 h post infusion. Blank biological fluid samples (fetal plasma, amniotic and tracheal fluid) were also obtained from near term pregnant sheep for use in validation studies (to determine whether the isomers can be separated from the endogenous substances within these fluids). The samples were stored at −20°C until analysis.

Sample preparation

Total concentration of labetalol was determined by the method of Yeleswaram *et al.* [1]. Briefly, to 50–500 μl of the plasma sample, the internal standard (SCH), HPLC-grade water (to 750 μl total volume) and 0.5 ml of pH 9.5 carbonate buffer (1 *M*) were added. The mixture was extracted with 6 ml of ethyl acetate. The ethyl acetate layer was separated and re-extracted with 600 μl of 0.01 *M* phosphoric acid. An aliquot (60 μl) of the aqueous layer was injected into the HPLC column. For determination of the individual isomers, aliquots of the samples were extracted by the procedure that was used in the labetalol assay but without the addition of the internal standard. The addition of the internal standard was not necessary since in the chiral assay the percentage of each isomer in the racemic mixture is determined and not the actual concentration. Since there is not enough of pure isomers available to construct a standard curve for each individual isomer on a routine basis, the achiral labetalol assay method previously described [1] is used to determine the total labetalol concentration in the samples, after which the concentration of each isomer is determined as described below. The concentration of each isomer is determined from the following relationship:

$$C_i = (\%\ \text{isomer} \times C_L)/100$$

where C_i = concentration of the individual isomer, % isomer = percentage of the individual isomer determined by the chiral assay and C_L = concentration of labetalol determined by the achiral assay [1].

Chromatographic conditions

The mobile phase was 0.02 *M* phosphate buffer containing 0.015 *M* TBAP. The mobile phase was degassed with helium for 30 min prior to the adjustment of the pH to 7.10 with phosphoric acid. The flow-rate was set at 0.5 ml/min. The separation of the isomers was accomplished at ambient temperature.

Assay characteristics

To obtain the concentration of individual isomers, the proportion of each isomer in the sample (equal to the peak area of the isomer divided by the total area of all four peaks) was multiplied by the total concentration of labetalol determined by the achiral assay. The average percentage peak area and coefficient of intra- and inter-day variation were calculated at 0.6, 1.5, 3.0, 6.0, 9.0, 12.0 and 15.0 ng of labetalol. Linear least-squares regression analysis was used to obtain the correlation coefficient for each isomer over the range 0.6–15.0 ng of injected labetalol. Resolution of the peaks, R_S, was calculated from the equation $R_S = (2t_{R1} - 2t_{R2})/(1.7W_{h1} + 1.7W_{h2})$ where t_R and W_h are retention time and peak width at half height, respectively, and 1 denotes the peak with longer retention time. Minimum quantifiable limit was defined as four times the baseline noise. The coefficient of variation (C.V.) was calculated as: C.V. = $100 \cdot \sigma/\chi$ where σ and χ are standard deviation and mean values, respectively.

RESULTS AND DISCUSSION

Chromatography

A typical chromatogram obtained following injection of 6.0 ng of labetalol is shown in Fig. 1A. Baseline separation defined as $R_S \geq 1.5$ was achieved by manipulation of the pH, TBAP concentration and flow-rate of the mobile phase. The retention times for *SR*, *SS*, *RS* and *RR* peaks were 19, 23, 28 and 34 min, respectively. The identity of the isomer peaks was established with the aid of authentic standards of which 1.5 ng of each were injected. The chromatograms of the individual isomers are shown in Fig. 1B–E.

The separation of the *SR* and *SS* isomers was particularly sensitive to the change in the pH of the mobile phase while the separation of the *RS* and *RR* isomers was more sensitive to changes in the concentration of the TBAP. At a pH of 7.10 and a TBAP concentration of 0.005 *M*, the *SR* and *SS* isomers eluted as separate peaks (R_S = 1.6) but the *RS* and *RR* isomers coeluted (Fig. 2A) while at pH 6.9 and a TBAP concentration of 0.015 *M* the *RS* and *RR* peaks were completely separated (R_S = 1.7) but *SR* and *SS* peaks were not (Fig. 2B). Changes in the pH and concentration of the cationic modifier in the mobile phase have been shown to greatly influence the enantioselectivity and hence retention of several different classes of drugs by AGP [12–14]. While changes in the pH of the mobile phase affect enantioselectivity by altering the conformation of the protein stationary phase [12–14], cationic modifiers such as TBAP and TBABr influence chiral separation through ion-pair formation and increase in the steric bulk of the molecule [6,7,14].

Calibration and reproducibility

Calibration characteristics corresponding to injection of 0.6–15 ng of racemic labetalol stock solution are shown in Table I. The percentage peak area of the isomers and the C.V. over the range of the amount of labetalol injected are shown in Table II. The C.V. ranged from 0.23 to 5.48%. The limit of quantitation (signal-to-noise ratio = 4) was 0.6 ng of labetalol or 0.15 ng of each isomer injected. The maximum amount of

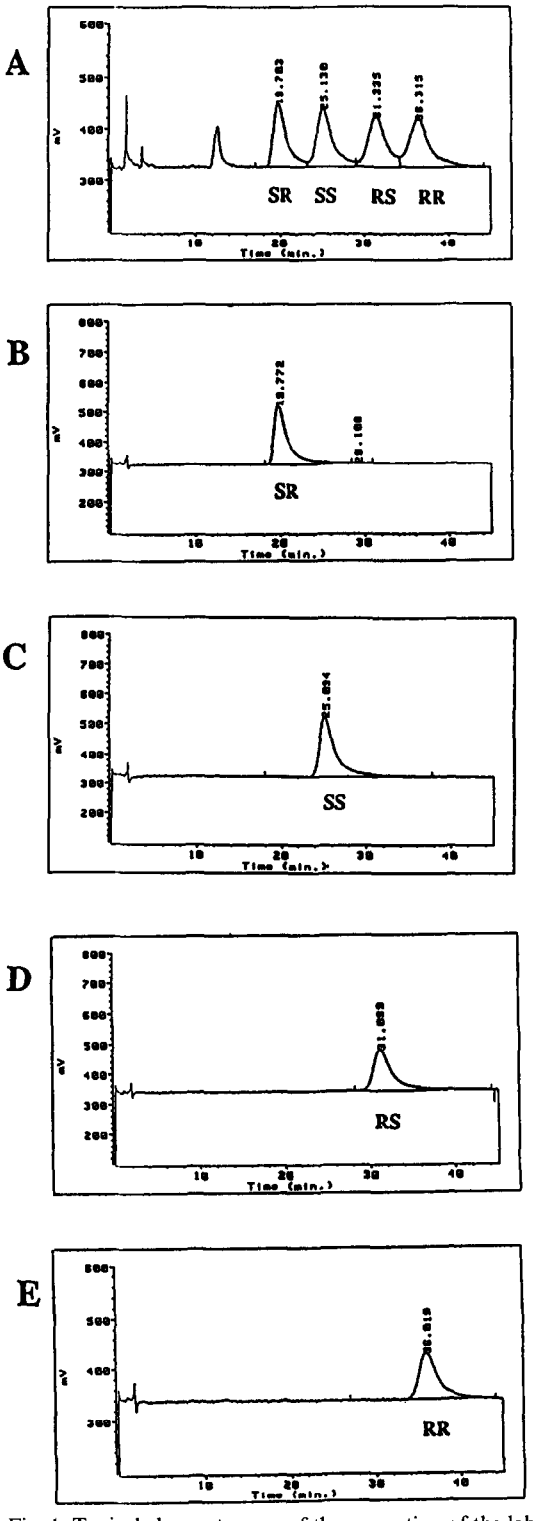

Fig. 1. Typical chromatogram of the separation of the labetalol stereoisomers (A) following the injection of 6.0 ng of racemic labetalol, and chromatograms of the individual isomers *SR* (B), *SS* (C), *RS* (D) and *RR* (E). pH = 7.1, 0.015 *M* TBAP.

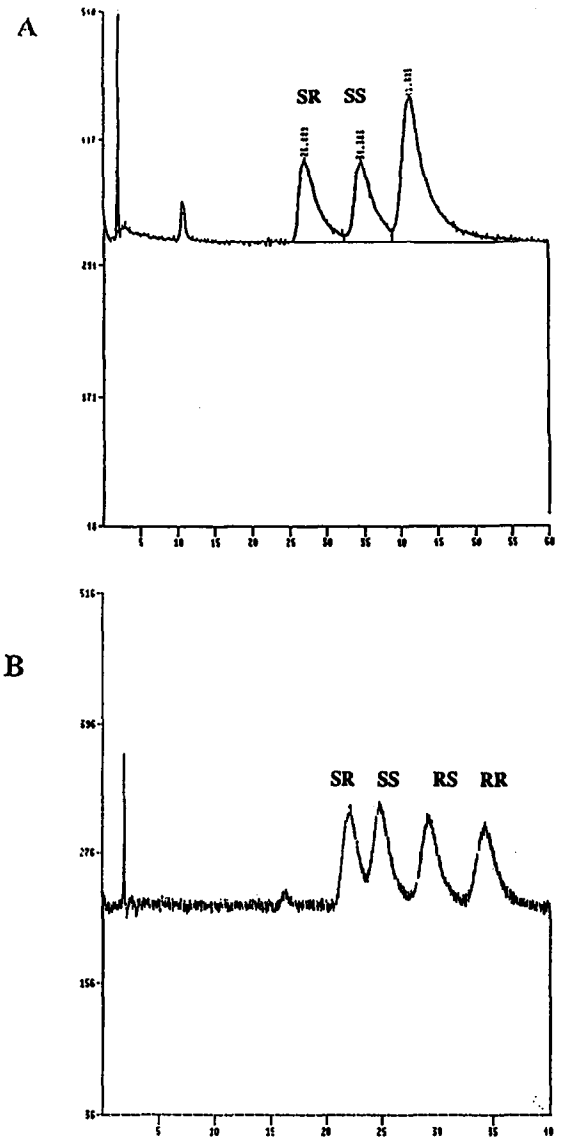

Fig. 2. Effects of pH of the mobile phase and TBAP concentration on the separation of labetalol stereoisomers. (A) pH = 7.1, 0.005 *M* TBAP. (B) pH = 6.9, 0.015 *M* TBAP.

labetalol that could be resolved at the baseline was 15.0 ng injected.

Determination of labetalol isomers in biological fluids

Labetalol was extracted from plasma obtained from chronically instrumented non-pregnant sheep and from blank fluids, spiked with labetalol, obtained from pregnant sheep from previous experiments. Sample chromatograms from blank fetal tracheal, maternal plasma and amniotic

TABLE I

CALIBRATION OF LABETALOL ISOMERS (n = 9)

Isomer	Correlation coefficient	Slope (mean ± S.D.)	Intercept (mean ± S.D.)
SR	0.9998	0.221 ± 0.001	0.009 ± 0.018
SS	0.9999	0.261 ± 0.001	−0.001 ± 0.011
RS	0.9999	0.265 ± 0.001	−0.006 ± 0.008
RR	0.9997	0.252 ± 0.002	−0.003 ± 0.025

TABLE II

AVERAGE PERCENTAGE PEAK AREA AND INTER- AND INTRA-DAY COEFFICIENTS OF VARIATION FOR EACH ISOMER FOLLOWING INJECTION OF RACEMIC LABETALOL (n = 9)

Amount of labetalol (ng)		*SR*	*SS*	*RS*	*RR*
0.6	Peak area	22.80	26.00	26.28	24.92
	Inter-day C.V.	5.48	0.85	2.93	4.01
	Intra-day C.V.	7.72	6.45	7.56	4.24
1.5	Peak area	22.17	25.90	26.38	25.55
	Inter-day C.V.	2.97	0.53	1.92	0.79
	Intra-day C.V.	6.81	2.49	1.73	3.48
3.0	Peak area	22.31	26.40	26.15	25.14
	Inter-day C.V.	2.36	1.87	2.42	2.52
	Intra-day C.V.	2.12	1.89	2.50	3.23
6.0	Peak area	22.42	25.75	26.46	25.36
	Inter-day C.V.	0.47	0.70	0.50	0.72
	Intra-day C.V.	1.29	0.78	1.42	1.96
9.0	Peak area	22.57	26.21	26.63	24.59
	Inter-day C.V.	0.88	0.76	1.33	2.34
	Intra-day C.V.	1.85	0.98	0.59	1.59
12.0	Peak area	22.25	26.16	26.43	25.17
	Inter-dayn C.V.	2.97	1.32	1.04	1.96
	Intra-day C.V.	2.17	0.91	1.39	1.03
15.0	Peak area	22.04	26.06	26.58	25.31
	Inter-day C.V.	2.01	0.22	0.70	1.94
	Intra-day C.V.	1.68	0.86	0.69	1.33

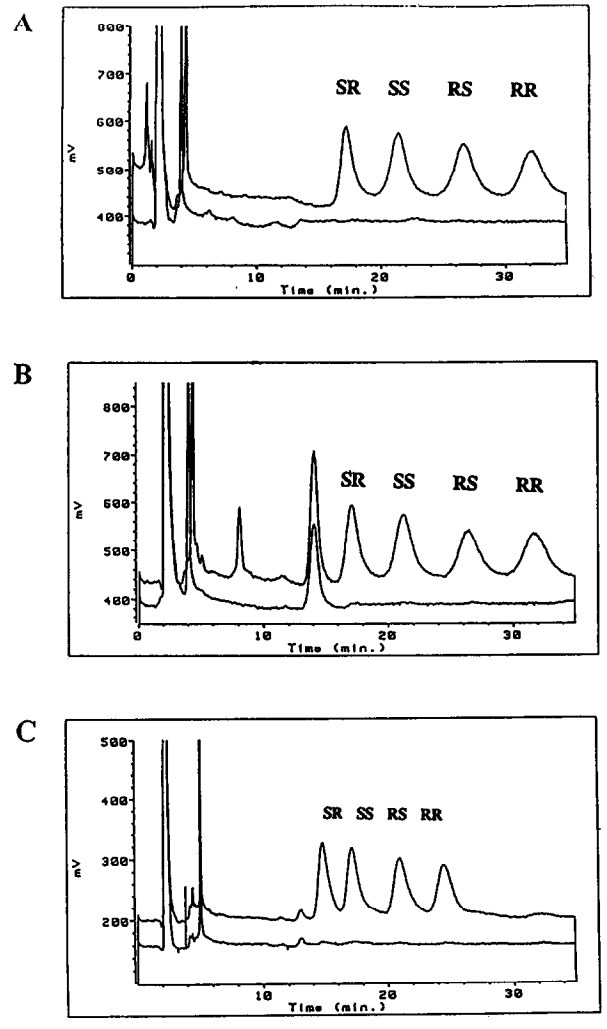

Fig. 3. Superimposed chromatograms of blank and spiked biological fluids. (A) Fetal tracheal fluid; (B) maternal plasma; (C) amniotic fluid.

fluid spiked with labetalol are shown in Fig. 3A, B and C, respectively. The chromatogram of isomers extracted from plasma obtained from non-pregnant sheep during post-infusion is shown in Fig. 4. The concentrations of the *SR*, *SS*, *RS* and *RR* isomers at 2 h post infusion were 12.7, 16.22, 13.38 and 19.70 ng/ml, respectively. The four isomers were well separated from the endogenous substances in all the fluids examined (plasma, tracheal and amniotic fluid).

Plasma samples from a non-pregnant ewe in-

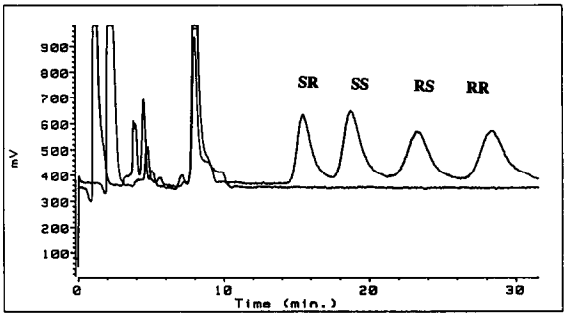

Fig. 4. Superimposed chromatograms of control plasma (blank) and plasma obtained at steady state from chronically instrumented non-pregnant sheep.

jected with a 100-mg bolus followed by a 0.5 mg/min infusion of labetalol were analyzed. For each sample the percentage of each isomer was calculated. Peak area of each isomer ranged from 22.0 and 28.0% of the total peak area of the four isomers. The concentration of each isomer was calculated as described above. The plot of concentration of labetalol and concentration of each isomer against time is shown in Fig. 5. For the non-pregnant sheep there does not appear to be any stereoselectivity in the disposition of labetalol isomers from the analysis of plasma samples obtained before steady state, during steady state and post infusion. To determine whether labetalol isomers undergo stereoselective disposition more samples are needed for statistical analysis (future studies).

CONCLUSIONS

A direct and sensitive HPLC assay for the separation of labetalol stereoisomers was developed. Baseline separation of the four stereoisomers of labetalol was achieved on an AGP stationary phase column. The four isomers in order of elution were *SR*, *SS*, *RS* and *RR* with retention times of 19, 23, 28 and 34 min, respectively. The limit of reliable detection of each isomer using 50–500 μl of biological fluid was 0.15 ng (0.6 ng of racemic labetalol injected). While there was no apparent evidence of stereoselective disposition

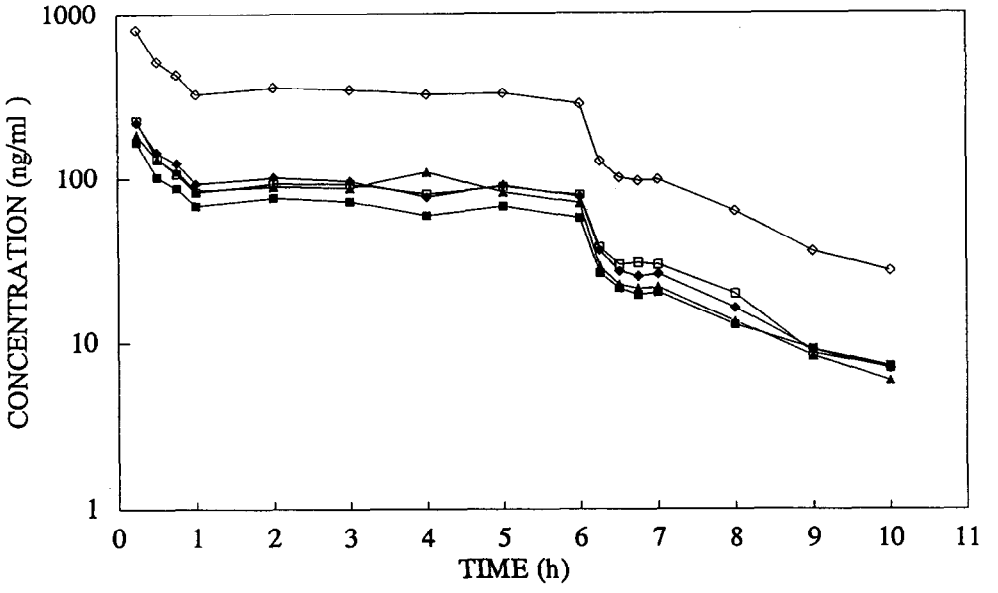

Fig. 5. Concentration–time profiles of labetalol (◇), *SR* (■), *SS* (◆), *RS* (▲) and *RR* (□) isomers in plasma obtained frm non-pregnant sheep administered with a 100-mg intravenous bolus of labetalol followed by a 0.5 mg/min infusion for 6 h.

of labetalol isomers in plasma samples obtained from a single non-pregnant sheep, further experimentation is required to examine this observation. The assay will be used to further study the characteristics of the stereospecific metabolism and disposition of labetalol in pregnant and non-pregnant sheep.

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