Pergamon

0731-7085(94)00077-8

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 11, pp. 1453–1461, 1994
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0731-7085/94 $7.00 + 0.00

Development and validation of a chiral LC method for analysis of the four stereoisomers of 1045U85 in plasma

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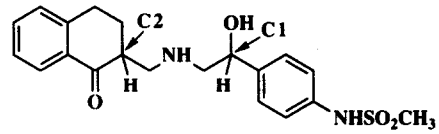
Abstract: A method for analysis of the four stereoisomers of 1045U85 in rat plasma was developed and validated. The method involved liquid extraction of 1045U85 and an internal standard (propranolol) from plasma, followed by reaction with a chiral derivatizing reagent, GITC. The diastereoisomeric products were then separated by reversed-phase LC. The range of quantitation was 9.828–0.121 μg ml^{-1} for total 1045U85 (3.440–0.042 μg ml^{-1} for the *RR* and *SS* isomers, and 1.474–0.018 μg ml^{-1} for the *RS* and *SR* isomers). Specificity of the method for 1045U85 was demonstrated using spiked plasma samples as well as plasma samples from dosed animals. Extraction recovery of 1045U85 and propranolol was greater than 95%, and the derivatization reaction was shown to be complete. Accuracy (% bias) ranged from −2.6 to 3.9% for total 1045U85 and from −4.7 to 14.1% for the individual stereoisomers. Precision (% RSD) was 3.8–8.7% for total 1045U85 and 2.9–16.5% for the individual isomers. Plasma samples stored at −70°C were stable for 19 weeks. The method has been used to determine plasma 1045U85 concentrations in nonclinical studies with this compound.

Keywords: *Derivatization; stereoisomers; enantiomers; indirect chiral LC assay; epimerization; pharmacokinetics; GITC.*

Introduction

Compound 1045U85 HCl (4′-1-hydroxy-2-(((1,2,3,4-tetrahydro-1-oxo-2-naphthyl)-methyl)amino)ethyl)methanesulphonanilide hydrochloride (Fig. 1) is undergoing evaluation as a potential antihypertensive agent. The compound contains two chiral carbon atoms, designated C1 and C2, and is a mixture of two pairs of enantiomers. All four stereoisomers are pharmacologically active (A. Tadepalli, BW Co., personal communication). The configuration at C1 is stable *in vitro* and *in vivo*; however, the configuration at C2 readily epimerizes *in vivo* and, under physiological conditions of temperature and pH, *in vitro*.

The instability of the configuration at C2 presented challenges beyond those of developing a chiral separation. The sample work-up had to eliminate or minimize configuration change at C2, so that concentrations of the four stereoisomers would not be altered as an artifact of the method. The method chosen involved extraction of plasma samples with an ethyl acetate solution containing the chiral derivatizing reagent, GITC (2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate).



1045U85

In Solution:

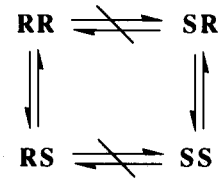


Figure 1
The structure of 1045U85 is shown at the top, with the chiral centres indicated at carbon atoms C1 and C2. 1045U85 is a racemate comprising four stereoisomers with configurations of *RS*, *RR*, *SS* and *SR* (configuration at C1 followed by C2). Chemical inversion of configuration occurs at C2 in aqueous media at physiological pH, resulting in equilibration between pairs of diastereoisomers but not enantiomers, as shown at the bottom.

GITC reacts readily with primary or secondary amines under very mild conditions, and early reports of its use by Nimura and coworkers demonstrated its value in separating enantio-

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mers of amino acids and catecholamines [1–3]. Since then, various investigators have used GITC as a chiral derivatizing reagent to form diastereoisomeric derivatives of propranolol and other beta blockers [4], ephedrine, norephedrine and pseudoephedrine [5], various amphetamines [6], epinephrine [7, 8], various adrenergics and other aminoalcohols [9, 10], and chloramphenicol and thiamphenicol [11]. This approach has also been applied to analysis in biological fluids of racemates or enantiomers of mexiletine [12], and propranolol and its metabolites [13, 14]. GITC reacted rapidly with 1045U85 under mild conditions, and gave derivatives that could be baseline separated when analysed by LC. The method used here differed from many other derivatization procedures in one important aspect: instead of having separate extraction and derivatization steps, in this procedure the GITC was dissolved in the extracting solvent. This approach permitted the derivatization reaction to take place while the analyte was being extracted from the biological sample. The derivatized reaction products were significantly more stable than the stereoisomers themselves, enabling analysis within 24 h after their preparation. Low pH (pH 4 or lower) and low temperature significantly retarded the epimerization at C2, and these conditions were employed wherever possible in the method. The development, validation and application of this method are described in this article.

Experimental

Materials and reagents

Various batches of 1045U85, composed of different percentages of the four stereoisomers, were prepared by staff in the Division of Organic Chemistry and the Chemical Development Laboratories, Burroughs Wellcome Co. The percentages of the stereoisomers in each batch were assigned based on the known stereochemistry of the starting materials, and the analysis of each batch by a LC method capable of resolving the diastereoisomers (but not the enantiomers; i.e. *RR* and *SS* coeluted at a different retention time than *RS* and *SR*). GITC (stereochemical purity = 100%) was obtained from Polysciences, Inc., Warrington, PA, USA. Normal rat plasma (NRP) was obtained from Pel-Freez Biologicals, Rogers, AR, USA. R-(+)-propranolol was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI, USA. ^{3}H-S-(−)-propranolol·HCl was purchased from NEN Research Products (DuPont Co., Wilmington, DE, USA). All solvents were LC grade (EM Science, Gibbstown, NJ, USA).

Stock solutions of 1045U85 and related compounds, and propranolol, were prepared in 1 mM HCl (to improve the stability of 1045U85). For each assay, two independent sets of standards were prepared by serial dilution of the 1045U85 stock solution in NRP, for a concentration range of 9.828–0.121 μg ml^{-1} (as free base). Quality control samples were prepared at concentrations near the high, middle and low range of the standard curve using a 1045U85 stock solution different from that used for standard curve preparation. Determinations of specificity, recovery, freeze/thaw stability, and stability of processed samples were performed using quality control samples, as well as plasma from dosed animals.

Early experiments demonstrated that epimerization at the C2 position occurred within 5 min after addition of 1045U85 to blank rat plasma at room temperature. Therefore, to minimize epimerization, blank plasma, standards, quality control samples, and samples from dosed animals were kept on ice up to the extraction/derivatization step. Stock solutions were used at room temperature. Samples, controls and stock solutions were stored at approximately −70°C when not being analysed. Stock solutions were stored for no longer than 1 month.

All standards, controls, and samples were processed as follows. A 0.5-ml volume of plasma was pipetted into an extraction tube, followed by 50 μl (1.25 μg) of R(+)propranolol as an internal standard, and 2 ml of ethyl acetate containing 50 μg ml^{-1} of the derivatizing reagent, GITC. The extraction tubes were capped, removed from the ice bath, and placed on a shaker for 10 min. Tubes were removed from the shaker and allowed to stand at room temperature for a minimum of 10 min, then centrifuged to separate the aqueous and organic layers. The ethyl acetate layer (top layer) was quantitatively transferred to a glass tube and blown to dryness under a stream of nitrogen. Dried samples were reconstituted with 200 μl of 50% acetonitrile in 1 mM HCl and filtered (0.45 μm) into injection vials. Volumes of 100 μl were injected onto the LC system.

LC conditions

The LC system consisted of Constametric I and IIIG Metering Pumps (LDC Analytical, Riviera Beach, FL, USA), an ISS 100 auto-injector (Perkin–Elmer Corp., Norwalk, CT, USA), and a Spectroflow 783 Programmable Absorbance Detector (ABI Analytical, Kratos Division, Ramsey, NJ, USA). Instrument control and data acquisition were by a DS 802 microcomputer (Digital Specialties, Carrboro, NC, USA). The method employed a Zorbax ODS (80 × 6.2 mm, 5 μm i.d.) column (MAC-MOD Analytical, Inc., Chadds Ford, PA, USA), preceded by a 30 × 4.5 mm guard column with 10 μm C18 packing (PhaseSep, Norwalk, CT, USA). Mobile phase A was methanol–water, 61:39 (v/v), and mobile phase B was methanol. A gradient of 100% mobile phase A for 700 s, followed by a linear ramp to 90% mobile phase B over 300 s was used, at a flow rate of 1 ml min^{-1}. Total run time was 1200 s. The column was purged at 2 ml min^{-1} with 90% mobile phase B for 180 s following each run, and was restored to 100% mobile phase A for 300 s at 1 ml min^{-1} prior to each subsequent run. Detection was by UV at 252 nm. For experiments determining the specificity of the method, some samples were analysed by the above chromatography methods, with detection of eluting material made with a model 991 photodiode array detector (Waters, Milford, MA, USA) across the wavelength range of 220–340 nm.

LC–MS analysis

Three analytical columns (described above) were connected in series, to maximize separation of the derivatized peaks. Mobile phase A was pumped at 0.5 ml min^{-1} using a Waters 600MS liquid chromatograph with a Waters 484MS UV detector set at 254 nm. The LC flow was coupled through a Nermag/Vestec 740C thermospray interface to a Nermag R10-10C quadrupole mass spectrometer, operated at a scan speed of 0.4 s per scan and a mass range of 150–850 amu with unit resolution over the mass range. A post-column flow of 0.5 ml min^{-1} of 95% 0.05 M ammonium acetate and 5% acetonitrile was used to aid the thermospray process. The data were analysed using a SIDAR data system.

Analysis

Integration limits for each of the GITC derivatives of the 1045U85 stereoisomers and the internal standard were set manually. A separate standard curve was constructed for each of the four stereoisomers, by weighted (1/concentration) least-squares linear regression of stereoisomer concentration against the ratio of the derivatized stereoisomer peak area/derivatized internal standard peak area. Concentrations of each of the four 1045U85 stereoisomers in unknowns were calculated from their own standard curves.

Results

Peak identification and specificity

Different batches of 1045U85, each with differing percentages of the four stereoisomers, as well as preparations containing only two of the stereoisomers, were used in developing and validating the assay (Table 1). The GITC-derivatized stereoisomers of 1045U85 eluted in the order of *RS*, *RR*, *SS*, and *SR* (the configuration at C1 followed by that at C2) (Fig. 2). For all batches analysed, the percentages of the stereoisomers, calculated by dividing the peak area of each deriv-

Table 1
Specificity of the method for the stereoisomers of 1045U85

	Stereoisomer percentages*							
	Nominal				Measured			
Compound	*RS*‡	*RR*	*SS*	*SR*	*RS*	*RR*	*SS*	*SR*
1045U85	18	32	32	18	18	32	32	18
1045U85	15	35	35	15	14	35	36	16
668U88	5	95	0	0	5	95	0	0
1421U87	48	52	0	0	47	53	0	0
1420U87	0	0	95	5	0	0	95	5
1419U87	0	0	51	49	0	0	52	48

*Percentages of stereoisomer derivatives are rounded to the nearest whole number.
†Compounds 668U88, 1421U87, 1420U87 and 1419U87 are separately synthesized mixtures of diastereoisomers.
‡Configuration at C1 followed by C2.

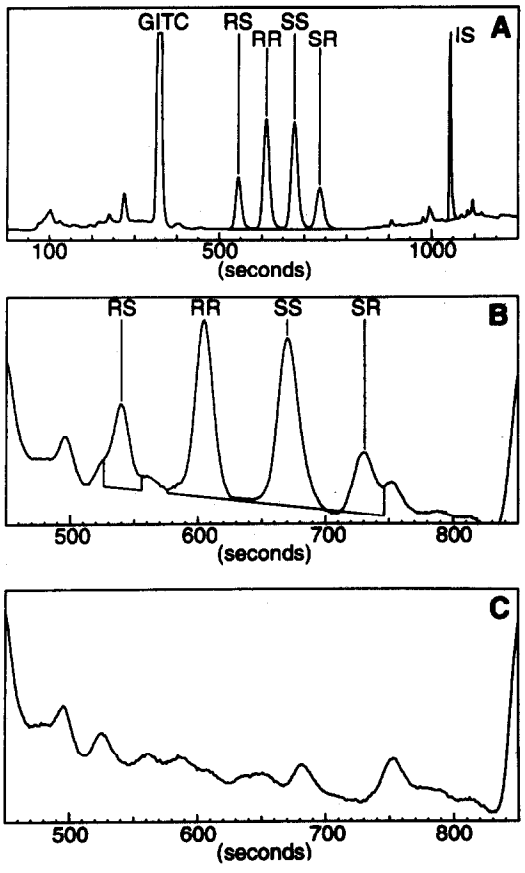

Figure 2
Chromatograms of blank rat plasma spiked with the internal standard and the highest concentration of 1045U85 used in the standard curve (A), the lowest concentration of 1045U85 used in the standard curve (B), or no added 1045U85 (C), after extraction and derivatization with GITC. Only the region of the chromatogram where the derivatized 1045U85 stereoisomers elute is shown in panels (B) and (C).

atized stereoisomer by the sum of the four peak areas, agreed with expected percentages to within 1% (Table 1).

Figure 2(A) shows the entire chromatogram for rat plasma spiked with the internal standard, and the highest concentration of 1045U85 used in the standard curve (9.828 μg ml^{-1}). Figure 2(B) is the chromatogram of the lowest standard concentration used in the curve in rat plasma (0.121 μg ml^{-1}), expanded to show the region containing the stereoisomer peaks. Comparison of the low standards to blank rat plasma (Fig. 2C) showed that there were no major interfering background peaks in blank plasma. However, there was a small background peak which eluted just after the *SR* stereoisomer.

UV spectra of the GITC-derivatives of each of the four stereoisomers of 1045U85 in NRP were obtained using photodiode array detection. The spectra of the GITC derivatives of all four 1045U85 stereoisomers were superimposable whether this analysis was performed on blank rat plasma spiked with 1045U85, or on plasma from rats dosed with the compound, indicating that the derivatized stereoisomer peaks did not contain detectable amounts of co-eluting components or metabolites (data not shown). Peak purity (specificity) was confirmed by LC–MS analysis of plasma samples from dosed animals. The molecular weight (mw) of each of the four 1045U85 diastereoisomer peaks was determined to be 777 daltons (1045U85 mw of 388 plus GITC mw of 389), and this was the only major ion observed at the retention time of each of the four derivatized 1045U85 peaks.

Selection of calibration model

The appropriate calibration curves for quantitation of 1045U85 stereoisomers were determined by evaluation of data from five sets of seven standards, prepared in NRP over the range of 29.484–0.040 μg ml^{-1} (concentrations represent the total of the four stereoisomers). Standards were prepared with an 18:32:32:18 (*RS*:*RR*:*SS*:*SR*) batch of 1045U85, which of all available batches, contained the four stereoisomers in the most equivalent proportions. (No batch of 1045U85 was available in which each stereoisomer represented 25% of the total). The range of linearity and LOQ for each of the stereoisomers were determined based on the examination of residuals. The data were best described by a weighted (1/C) linear regression using standards ranging from 9.828 to 0.121 μg ml^{-1}. The mean concentrations for each standard, obtained by back-calculating the peak area/internal standard area ratios against the corresponding standard curve, are shown in Table 2. The percentage bias indicates that back-calculated concentrations agreed very well with the nominal concentrations for all standards. RSD values in all cases except for the *SR* stereoisomer at the lowest concentration tested, were below 10% (the background peak eluting near the retention time of the *SR* isomer, as mentioned above, may have contributed to the higher % RSD at low concentrations of *SR*). Therefore, the range of linearity was determined to be 1.769–0.022 μg ml^{-1} for the *RS* and *SR* stereoisomers, and 3.145–0.039 μg ml^{-1} for the *RR* and *SS* stereoisomers. The LOQ for each

Table 2
Back-calculated values for standards after weighted (1/C) linear regression of the standards*

Stereoisomer	Nominal conc. μg ml^{-1}	Mean† conc. μg ml^{-1}	SD	% RSD	% Bias
RS	1.769	1.786	0.007	0.4	1.0
	0.590	0.583	0.027	4.6	−1.2
	0.197	0.187	0.010	5.3	−5.1
	0.066	0.065	0.001	1.5	−1.5
	0.022	0.023	0.002	8.7	4.5
RR	3.145	3.172	0.137	4.3	0.9
	1.048	1.037	0.051	4.9	−1.1
	0.349	0.333	0.015	4.5	−4.6
	0.116	0.113	0.006	5.3	−2.6
	0.039	0.042	0.002	4.8	7.7
SS	3.145	3.169	0.145	4.6	0.8
	1.048	1.038	0.054	5.2	−1.0
	0.349	0.334	0.018	5.4	−4.3
	0.116	0.115	0.009	7.8	−0.9
	0.039	0.041	0.004	9.8	5.1
SR	1.769	1.779	0.081	4.6	0.6
	0.590	0.585	0.024	4.1	−0.8
	0.197	0.192	0.009	4.7	−2.5
	0.066	0.066	0.001	1.5	0.0
	0.022	0.023	0.004	17.4	4.5

*The concentration range for total 1045U85 in standards was 9.828–0.121 μg ml^{-1}.
†$n = 5$.

Table 3
Percentage recovery of radioactivity from rat plasma spiked with ^{14}C-1045U85 or ^{3}H-propranolol

	Ethyl acetate		Vial		
	Mean	SD	Mean	SD	*n*
^{14}C-1045U85 recovery: spiked rat plasma					
(6.514)*	95.0	1.0	84.3	1.4	5
(0.651)*	95.5	1.2	79.1	5.3	4
(0.065)*	95.1	4.4	82.2	1.8	4
Dosed rat plasma	96.4	2.5	81.4	2.3	2
^{3}H-propranolol recovery: spiked rat plasma					
(8.962)*	98.6	1.0	81.0	1.1	4
Dosed rat plasma	99.8	2.3	80.5	1.4	4

*Values in parentheses indicate concentration of nonradiolabeled 1045U85 spiked into sample.

stereoisomer was set as the lowest concentration of the corresponding standard curve.

Recovery and completion of the derivatization reaction

Recovery was determined using ^{14}C-1045U85 and ^{3}H-propranolol (Table 3). Recovery of ^{14}C-1045U85 from spiked rat plasma averaged approximately 95% in the ethyl acetate extract and approximately 81% in the injection vial. There were no differences in extraction efficiency due to 1045U85 concentration. The recovery of ^{14}C spiked into plasma samples from dosed animals averaged 96% for the ethyl acetate extraction and 82% for the injection vial, showing no interference from potential metabolites or breakdown products. Recovery of ^{3}H-propranolol in the ethyl acetate extracts averaged approximately 100% from spiked control plasma and 99% from plasma of dosed animals, while recovery in the injection vial averaged 80% from both spiked controls and plasma from dosed animals.

A comparison of reaction times of 10, 30 and 60 min at room temperature (0.05 mg ml^{-1} GITC) showed no significant difference in the peak areas of 1045U85 or propranolol. A comparison of the effect of GITC concentration on peak areas, made by derivatizing spiked plasma with 0.05, 0.1, or 0.25 mg ml^{-1} of GITC in ethyl acetate for 30 min at room temperature, also indicated no significant differences in peak areas among the different GITC concentrations. Therefore, a minimum

10-min reaction time and the amount of GITC specified in the method (0.05 mg ml^{-1}) were adequate for maximum derivatization of the compounds over the concentration range of the standard curve.

Accuracy and precision

The intra- and inter-assay accuracy and precision of the method were determined by replicate analysis of control rat plasma spiked with 1045U85 at concentrations corresponding to the high, medium and low range of the standard curve (Table 4). No trends were observed in the intra-assay accuracy and precision of the method due to 1045U85 concentration. Intra-assay accuracy, expressed as the % bias, ranged from −2.6 to 2.3% for quantitation of total 1045U85 in plasma and from −4.7 to 14.1% for quantitation of the individual stereoisomers. Intra-assay precision, expressed as the % RSD, ranged from 3.8 to 5.4% for total 1045U85 and from 2.9 to 10.0% for the individual stereoisomers.

Duplicate analysis of spiked rat plasma, daily over 5 days, showed an inter-assay accuracy ranging from −2.0 to 3.9% for quantitation of total 1045U85 and −0.5–13.1% for quantitation of the individual stereoisomers. The inter-assay precision ranged from 3.9 to 8.7% for total 1045U85 and 3.7–16.5% for individual stereoisomers.

Stability of 1045U85 in plasma stored at −70°C

The stability of 1045U85 in rat plasma stored at approximately −70°C was monitored over 19 weeks. There were no obvious trends in the concentration values for total 1045U85 or for the individual stereoisomers in plasma of either species, indicating that the stereoisomers of 1045U85 were stable at −70°C over the time spans examined.

In addition to analysis of spiked plasma stored at −70°C, 26 plasma samples from dosed rats were analysed before and after storage for 8 months at −70°C. The median difference between concentrations of paired

Table 4
Accuracy and precision of assay

Spike	Isomer	Nominal μg ml^{-1}	Mean μg ml^{-1}	SD	Accuracy (% Bias)	Precision (% RSD)
Intra-assay*						
High	*RS*	0.921	0.980	0.029	6.4	2.9
	RR	2.764	2.773	0.107	0.3	3.8
	SS	2.764	2.787	0.115	0.8	4.1
	SR	0.921	1.001	0.038	8.6	3.8
	Total	7.371	7.541	0.287	2.3	3.8
Medium	*RS*	0.184	0.182	0.007	−1.2	3.8
	RR	0.553	0.527	0.033	−4.7	6.2
	SS	0.553	0.531	0.033	−3.9	6.3
	SR	0.184	0.195	0.007	6.0	3.8
	Total	1.474	1.435	0.078	−2.6	5.4
Low	*RS*	0.037	0.042	0.004	14.1	8.3
	RR	0.111	0.112	0.005	0.6	4.8
	SS	0.111	0.107	0.004	−3.5	4.1
	SR	0.037	0.038	0.004	2.1	10.0
	Total	0.295	0.299	0.014	1.3	4.6
Inter-assay†						
High	*RS*	1.115	1.166	0.045	4.6	3.8
	RR	2.601	2.625	0.114	0.9	4.3
	SS	2.601	2.629	0.111	1.1	4.2
	SR	1.115	1.161	0.044	4.1	3.8
	Total	7.432	7.580	0.307	2.0	4.0
Medium	*RS*	0.223	0.237	0.010	6.1	4.1
	RR	0.520	0.517	0.023	−0.5	4.5
	SS	0.520	0.519	0.019	−0.1	3.7
	SR	0.223	0.243	0.010	8.9	4.2
	Total	1.486	1.516	0.059	2.0	3.9
Low	*RS*	0.032	0.036	0.004	13.1	10.2
	RR	0.074	0.074	0.006	0.4	7.7
	SS	0.074	0.076	0.005	2.6	7.1
	SR	0.032	0.034	0.006	5.9	16.5
	Total	0.212	0.220	0.019	3.9	8.7

*n = 5 at each concentration.
†n = 10 at each concentration (two runs per day for 5 days).

samples was 6.5%. The relative percentages of the stereoisomers remained essentially constant. These results indicated that no major changes in 1045U85 concentrations occurred in plasma of dosed rats under the storage conditions stated above.

Freeze/thaw stability of 1045U85 in plasma

The stability of 1045U85 in plasma with repeated freezing and thawing was evaluated with spiked NRP, and plasma from two dosed rats. Spiked plasma samples were analysed immediately, and after each of five freeze/thaw cycles. Samples from dosed rats were removed from storage at approximately −70°C, thawed, and portions were analysed prior to and after each of four freeze/thaw cycles. No trends were observed in the stereoisomer concentrations or ratios (Table 5), indicating that 1045U85 underwent minimal degradation in plasma after multiple freeze/thaw cycles.

Stability of 1045U85 in plasma during sample preparation

Due to of the rapid epimerization at C2 at room temperature, samples were kept on ice up to the time of extraction with ethyl acetate and derivatization with GITC. The stability of 1045U85 in rat plasma over 3 h at 4°C was examined, to determine whether any change in stereoisomer percentages would occur at 4°C during the amount of time reasonably required for sample work-up. The results (Table 6)

Table 5
Stability of 1045U85 in rat plasma after freeze/thaw cycles*

Sample	Freeze/thaw cycle	Total conc.	% *RS*	% *RR*	% *SS*	% *SR*
Rat spike	0	5.669	15	35	35	15
(5.345 μg ml^{-1})	1	5.275	15	35	35	15
	2	5.262	15	35	35	15
	3	5.393	15	35	35	15
	4	5.255	15	35	35	15
	5	5.466	15	35	35	15
Dosed rat 1	0	5.962	19	25	25	31
	1	5.977	20	25	24	31
	2	4.968	20	25	24	31
	3	5.401	20	26	24	31
	4	5.587	19	25	24	31
Dosed rat 2	0	6.350	20	23	22	35
	1	6.208	20	23	22	36
	2	6.089	20	23	22	36
	3	5.873	20	23	22	36
	4	5.957	20	23	22	36

*All values for rat spikes are the mean of two observations. Values for dosed rats are a single observation.
†Nominal concentration, as total 1045U85.

Table 6
Stability of 1045U85 in rat plasma at 4°C

Sample*	Time† (h)	Total‡ area ratio	% *RS*	% *RR*	% *SS*	% *SR*
Rat high spike	0.0	2.187	14	35	37	15
	0.5	1.807	15	34	36	15
	1.0	2.067	15	34	36	16
	2.0	2.238	16	33	35	16
	3.0	2.168	16	33	34	17
Rat low spike	0.0	0.205	15	36	35	14
	0.5	0.198	16	35	34	15
	1.0	0.209	17	35	34	14
	2.0	0.206	17	34	34	15
	3.0	0.212	17	33	33	16

*Nominal concentrations, as total 1045U85, were 4.507 μg ml^{-1} for high spikes and 0.450 μg ml^{-1} for low spikes.
†Indicates amount of time the spiked plasma sample sat in ice before being derivatized and analysed.
‡All values are the ratio of the derivatized stereoisomer peak area/derivatized internal standard peak area.

indicated that total 1045U85 concentrations were quite stable at 4°C for up to 3 h. The stereoisomers present in plasma at the highest concentration (*RR* and *SS*) apparently epimerized slowly at C2, resulting in slightly lower concentrations of *RR* and *SS* and slightly higher concentrations of *RS* and *SR* by the end of the 3-h incubation period.

Assays of study samples were typically set up to allow all samples, standards, and external controls to be chromatographed within 24 h of processing. The stability of derivatized 1045U85 in processed rat plasma over 24 h at room temperature in the autoinjector was evaluated by replicate analysis of pooled samples. The concentration values from spiked rat plasma and plasma from a dosed rat (Table 7) indicated that 1045U85 was stable in processed plasma over 24 h.

Application

The method just described was used to analyse plasma samples obtained from rats given 30 mg kg^{-1} oral doses of 1045U85 by gavage. The rats were euthanized in groups of four at various time points after dosing. Figure 3 shows the resulting mean concentrations of each of the four stereoisomers, and their total, in the plasma of these rats. While the dosing material existed as a 15:35:35:15 (*RS*:*RR*:*SS*:*SR*) mixture of the four stereoisomers, this relationship was not observed in dosed rats at any of the time points analysed, due to epimerization *in vivo* at C2, and also perhaps due to possible stereoselectivity in the disposition of this compound in rats.

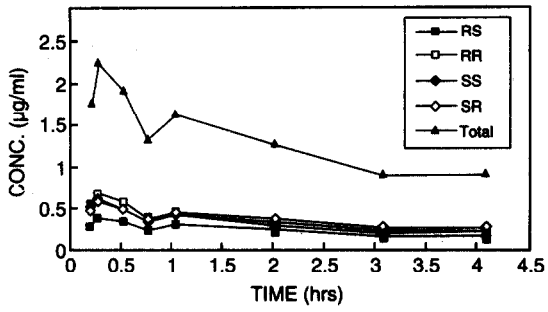

Figure 3
Mean plasma concentrations of the four stereoisomers of 1045U85, and their total, in rats given a 30 mg kg^{-1} oral dose of 1045U85.

Discussion

The facile epimerization at C2 in the 1045U85 molecule presented extraordinary problems with respect to its analysis in biological samples. Direct chiral methodology (i.e. use of a chiral column or chiral mobile phase additives) proved unsuccessful; despite repeated efforts, no such method attempted yielded the expected percentages of the stereoisomers. Several chiral derivatizing reagents were evaluated before selecting GITC. This compound reacted rapidly with 1045U85 under very mild conditions to form diastereoisomeric derivatives that were baseline separable when analysed by LC. Furthermore, its 100% stereochemical purity eliminated concerns over artifactual misrepresentation of 1045U85 stereoisomer amounts due to stereochemical impurities in the derivatizing reagent.

In the initial derivatization experiments, the

Table 7
Stability of derivatized 1045U85 in processed rat plasma over 24 h

Sample	Time* (h)	Total conc. µg ml^{-1}	% *RS*	% *RR*	% *SS*	% *SR*
High spike (8.235 µg ml^{-1})†	0	8.358	15	35	35	15
	10	8.490	15	35	35	15
	16	8.571	15	35	35	15
	24	8.439	15	35	35	15
Low spike (0.350 µg ml^{-1})†	0	0.328	15	34	36	15
	10	0.356	16	34	35	16
	16	0.346	16	35	34	15
	24	0.371	15	34	36	15
Dosed rat	0	1.788	17	20	25	38
	24	1.742	18	20	25	37

*Indicates the amount of time between the first injection and subsequent injections.
†Nominal concentration, as total 1045U85.

conventional approach to production of GITC derivatives was followed. That is, 1045U85 was first extracted from plasma into ethyl acetate, followed by evaporation of this organic solvent, and then treatment of the residue with a solution of GITC in acetonitrile. This procedure was only partially successful, in that four derivatives were formed, but not in the expected percentages. (For example, the batch of 1045U485 containing the *RS*, *RR*, *SS*, *SR* stereoisomers in an 18:32:32:18 ratio, was analysed at 22:26:28:24 by this approach). Modifying this procedure to include GITC in the extracting solvent enabled the derivatization to proceed simultaneously with the extraction, and as a result, epimerization was minimized and the correct percentages were measured. In addition, simultaneous extraction and derivatization resulted in a 30% increase in overall recovery of the derivatives, compared to the conventional approach, improving the lower limit of quantitation of the assay.

The specificity of the method was evaluated thoroughly, particularly after analysis of plasma samples from dosed animals. While it was expected that the relative proportions of the four stereoisomers in animal plasma would differ from their proportions in the dosing material, due to inversion of configuration at C2, it was also expected that the proportions of the enantiomers would be similar as a result of this inversion (for example, using dosing material in which proportions of *RS*:*RR*:*SS*:*SR* were 15:35:35:15, a proportion in plasma at some time after dosing might have been 20:30:30:20, if only chemical inversion were occurring). Instead, as shown in Fig. 3 and Tables 5 and 7, the relative proportions of the circulating stereoisomers in dosed rats were unequal. Out of concern that these results may have been due to derivatized metabolites or degradation products in the plasma co-eluting with the 1045U85 derivatives, samples from dosed rats were analysed using diode array detection and LC–MS methodologies. Both of these techniques verified the specificity of the method. Thus, the plasma concentrations in rats suggest a stereoselectivity in the disposition of 1045U85 in this animal. This stereoselectivity is a subject of further exploration.

The method has also been validated for use in analysing 1045U85 in plasma from dosed monkeys. Recovery, accuracy and precision, extent of derivatization reaction, and stability characteristics were essentially the same for monkey plasma samples as for those described above for rat plasma samples. Thus, the method is adaptable to more than one species.

Conclusions

An accurate, precise, specific indirect chiral LC method was developed for analysing the four stereoisomers of the novel potential antihypertensive agent, 1045U85, in plasma of rats. The method successfully minimized artifactual inversion of configuration at the readily epimerized chiral C2 position in the molecule. The resulting diastereoisomeric derivatives of 1045U85 enjoyed enhanced stability relative to the parent molecule. The method is suitable for use in non-clinical studies on the disposition of 1045U85 in rats and monkeys.

Acknowledgements — We are grateful to Dr Anjaneyulu S. Tadepalli, Dr John W.A. Findlay, Dr John McDermed and Mr Kevin Hurley for helpful discussions throughout this effort, and to Mr James Cichetti and Mr Pisal Chandrasurin for LC–MS analysis.

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[Received for review 9 March 1994; revised manuscript received 25 May 1994]