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Development of direct stereoselective and non-stereoselective assays in biological fluids for the enantiomers of a thieno[2,3-*b*]thiopyran-2-sulfonamide, a topically effective carbonic anhydrase inhibitor

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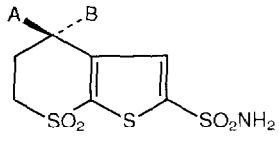
SUMMARY

A stereoselective assay for the optical isomers [(*S*) and (*R*)] of 5,6-dihydro-4-[(2-methylpropyl)amino]-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide-7,7-dioxide in human whole blood has been developed. The assay is based on direct enantiomer separation on a chiral stationary phase column of bovine serum albumin attached to silica. The effect of pH, ionic strength, column length and organic modifier on chiral separation has been studied. The assay methodology, based on high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (252 nm), has been fully validated in the concentration range 25–250 ng/ml of each enantiomer. Since no interconversion of the isomers was observed in vivo for the clinical studies involving the single (*S*)-enantiomer, a more sensitive (2.5 ng/ml), non-stereoselective assay has been developed. This method, also based on HPLC with UV detection, was fully validated in whole blood, plasma and urine in the concentration range 2.5–100 ng/ml. The details of these assays, together with some representative data from a pilot human study, are also presented.

INTRODUCTION

Racemic 5,6-dihydro-4-[(2-methylpropyl)amino]-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide-7,7-dioxide (compound **1**) and its (*S*)-enantiomer (Fig. 1) were found to possess good ocular hypotensive activity in various animal pharmacological models as well as in humans. These topically active and water-soluble carbonic anhydrase (CA) inhibitors have been selected for biochemi-



(S)-1, A = H, B = — $NHCH_2CH(CH_3)_2$

(R)-1, A = — $NHCH_2CH(CH_3)_2$, B = H

Fig. 1. Chemical structure of compound **1** and absolute configurations of its (*S*)- and (*R*)-enantiomers.

cal, pharmacological and clinical evaluation as potential antiglaucoma agents [1–6]. In order to support a clinical pharmacokinetic program, an assay of **1** and its enantiomers in biological fluids at low ng/ml levels was desired. Since the stereoselective disposition of the isomers of chiral drugs is a well established phenomenon in the field of pharmacokinetics and biopharmaceutics [7,8], and optical isomers usually exhibit differences in biological activity and in pharmacokinetic behavior, the stereoselective (SS) assay was initially required to study the disposition of the enantiomers when a racemic mixture of **1** was administered to human subjects. The SS assay was also needed to study the in vivo conversion of the (*S*)-**1** to (*R*)-**1** after similar administration of (*S*)-**1**. Since no conversion of the isomers was observed in vivo for clinical studies involving (*S*)-**1**, a more sensitive (2.5 ng/ml) but non-stereoselective (NSS) assay has been also developed.

Separation of enantiomers can be accomplished either directly or indirectly after derivatization to diastereomers with homochiral reagents. If possible, the direct mode is usually preferred [9]. For direct separation, either chemically bonded chiral stationary phases (CSPs) or conventional stationary phases with chiral mobile phase additives are used. Reviews on the direct chromatographic separation of enantiomers using these two approaches are available [10–15]. Most of the work on direct chiral separation has been focused on analytes in a simple sample matrix; little has been published on the direct separation of drug enantiomers in biological fluids [16–19]. The separation of the enantiomers of **1** based on this direct chiral approach, and development of the SS assay in whole blood and NSS assays in whole blood, plasma and urine, is the subject of this paper. The development of an SS assay for the enantiomers of **1**, based on an indirect approach and formation of diastereomers, is the subject of a separate paper [20].

EXPERIMENTAL

Reagents

HPLC-grade ethyl acetate, hexane, methanol, acetonitrile, water and toluene were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). Tri-

chloroacetic acid (TCA), phosphoric acid, 2-propanol, potassium hydroxide, sodium phosphates mono- and dibasic and copper sulfate were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The drug-free human whole blood and plasma (heparinized) were purchased from Biological Specialty (Lansdale, PA, U.S.A.). The drug standards [(*S*)-**1**, (*R*)-**1** and **1**] were obtained from Merck Sharp and Dohme Research Labs. (Rahway, NJ, U.S.A.). N-Benzoxycarbonyl-glycyl-L-proline (ZGP), a chiral mobile phase additive, was purchased from Fluka (Ronkonkoma, NY, U.S.A.).

Instrumentation

The Waters Assoc. 703 high-performance liquid chromatographic (HPLC) system, equipped with a 730 data module, a 720 system controller, a WISP 710B automatic injector (with limited-volume plastic inserts) and a 6000A chromatographic pump (Waters–Millipore, Milford, MA, U.S.A.), was used for all analyses. As a detector, the Spectroflow 773 UV detector (Kratos, Ramsey, NJ, U.S.A.) was utilized. Sensitivity was further enhanced by a Spectrum Model 1021A filter and amplifier (Newark, DE, U.S.A.). The detector output signals were interfaced to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, U.S.A.). The absorption spectra were taken using a diode array spectrophotometer (Hewlett-Packard 8452) and directly from HPLC runs using a photodiode array UV detector (Polychrom 9060, Varian, Walnut Creek, CA, U.S.A.). The following chiral HPLC columns were utilized in the exploratory part of the study: human α_1-acid glycoprotein (EnantioPac, 100 mm×4.6 mm I.D., LKB, Bromma, Sweden), (*S*)-N-(3,5-dinitrobenzoyl)leucine (Pirkle column, 250 mm×4.6 mm I.D., J.T. Baker, Phillipsburg, NJ, U.S.A.), β-cyclodextrin (100 mm×4.6 mm I.D., Advanced Separations Technologies, Whippany, NJ, U.S.A.), Chiralpak OC, Chiralcel CA-1 and Chiralpak WM (250 mm×4.6 mm I.D., Baker) and bovine serum albumin (BSA) attached to silica column (Resolvosil, BSA-7, 150 mm×4 mm I.D., Macherey-Nagel, Düren, F.R.G.). In the NSS assay, a Beckman reversed-phase (RP) non-end-capped C_8 column (250 mm×4.6 mm I.D., 5 μm, Beckman Instruments, Berkeley, CA, U.S.A.) and Brownlee Labs. RP C_8 guard column (RP-8 40 mm×4.6 mm I.D., 5 μm) were utilized.

Chromatographic conditions

The mobile phase in the SS assay was a mixture of 2-propanol–0.025 *M* phosphate buffer (pH 7.6) (1:99, v/v) delivered at a flow-rate of 0.5 ml/min. Two BSA-7 chiral columns in series were employed. In the NSS assay, the mobile phase was a mixture of acetonitrile–0.085% phosphoric acid (40:60, v/v) delivered at a flow-rate of 1.5 ml/min. The effluent from the column was monitored by the UV detector set at the wavelength of 252 nm. All mobile phase components were measured separately, mixed and filtered through a 0.2-μm nylon 66 filter (Rainin Instruments, Woburn, MA, U.S.A.).

In the exploratory work with various chiral columns, the mobile phases were as follows: EnantioPac, 2-propanol–0.008 *M* sodium phosphate monobasic (8:92, v/v) with or without 0.05 *M* sodium chloride, flow-rate 0.3 ml/min; Pirkle, hexane–2-propanol (90:10, v/v), flow-rate 0.5 ml/min; β-cyclodextrin, methanol–water (25:75, v/v), flow-rate 0.5 ml/min; Chiralpak OC, hexane–ethanol (90:10, v/v), flow-rate 0.5 ml/min; Chiralcel CA-1, ethanol–water (80:20, v/v), flow-rate 0.5 ml/min; Chiralpak WM, 0.25 *M* copper sulfate in water with up to 20% methanol, flow-rate 1.0 ml/min.

In the attempted separation using chiral mobile phase additive (ZGP), a LiChrosorb diol column (125 mm×3.9 mm I.D., 5 μm) and a mobile phase consisting of methylene chloride containing 3.5 m*M* ZGP was utilized.

Standard solutions

A stock standard solution of **1** (1 mg/ml) or (*S*)-**1** was prepared in methanol by weighing 11.08 mg of the hydrochloride salts and diluting with solvents to 10 ml. These solutions were further diluted in the same solvent to give a series of working standards with concentrations of 100, 10, 5, 1, 0.5 and 0.25 μg/ml. Stock solutions were stored at −5 °C and kept for a period of up to two weeks.

Sample preparation

Whole blood. The whole blood specimens were thawed to room temperature and vortex-mixed. Whole blood (0.5 ml) was placed in a 15-ml polypropylene centrifuge tube together with 0.5 ml of water (to lyse red blood cells, RBCs), 200 μl of 10% TCA and 10 μl of methanol. After vortex-mixing for 5 min, the phosphate buffer (3.5 ml, 0.05 *M*, pH 8.0) was added and the contents vortex-mixed for another 5 min. The sample was extracted with 5 ml of extraction solvent (toluene–ethyl acetate–2-propanol, 49:50:1, v/v) by shaking for 10 min at 120 strokes/min followed by centrifugation for 5 min at 5000 *g*. The organic phase was transferred to a new polypropylene tube (15 ml) and the aqueous phase extracted the second time. The combined organic phases (total of 9 ml) was then back-extracted with 0.3 ml of 0.025 *M* phosphoric acid by shaking the contents for 10 min and centrifugation (5 min at 5000 *g*). The organic phase was aspirated off and 200 μl of the acidic portion were injected directly into the HPLC system. In the case of the SS assay, the acidic portion after back-extraction was neutralized first to pH 7.0 with 10 μl of 0.5 *M* sodium hydroxide before injection on chiral BSA-7 columns.

Plasma. The plasma specimens were thawed to room temperature, vortex-mixed and centrifuged for 5 min at 5000 *g*. Plasma (0.5 ml), methanol (10 μl) and 200 μl of 10% TCA were transferred to a polypropylene centrifuge tube (15 ml). From this point, the procedure was the same as in whole blood.

Urine. The urine specimens were thawed to room temperature and vortex-mixed. An 0.5-ml aliquot containing 10 μl of methanol was extracted twice with

5 ml extracting solvent, after the addition of 1 ml of phosphate buffer (0.05 *M*, pH 8). The smaller volume of buffer (1 ml instead of 3.5 ml) was sufficient to adjust the pH to 8.0 since the protein precipitation step with 200 μl of 10% TCA was not required. From this point, the procedure was the same as in whole blood.

Quantification

A standard curve of **1** in the appropriate biological fluid was constructed and assayed daily with the unknown samples by plotting peak heights (NSS assay) or peak areas (SS assay) versus concentration of **1** or (*S*)-**1** and (*R*)-**1**. Two separate standard curves for (*S*)-**1** and (*R*)-**1** were constructed by spiking control human whole blood with known concentrations of **1** (as a racemic mixture) over the range 25–250 ng/ml for each enantiomer. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted linear regression of the standard curve. The weight was set to equal the inverse of the variance at each concentration. The order of elution of (*S*)-**1** and (*R*)-**1** was ascertained by monitoring the retention times of separately injected enantiomers.

Precision, accuracy, selectivity and recovery

The precision of the methods was determined by replicate analyses ($n = 5$) of a biological fluid containing **1** at all concentrations utilized for constructing calibration curves. A series of quality control (QC) standards were prepared in whole blood, plasma and urine at concentrations 5 and 50 ng/ml (NSS assay). These QC samples were prepared by transferring 50 μl of 10 μg/ml or 50 μl of 100 μg/ml standards of **1** to a 100-ml volumetric flask and diluting to mark with control whole blood, plasma or urine. The accuracy of the assay was expressed by (mean observed concentration)/(expected concentration) × 100.

The assay selectivity was assessed by running blank control and various patients' pre-dose whole blood, plasma and urine samples. No endogenous interference was encountered.

The recovery was determined by running standard curves from biological fluids and comparing these curves directly with the standard line obtained by injecting known amounts of available standards.

RESULTS

Direct separation on chiral CSPs

The best separation of enantiomers of **1** was achieved on protein-bonded phase consisting of BSA covalently bonded to silica gel. The separation on BSA-7 columns is illustrated in Fig. 2.

Almost baseline resolution of enantiomers was achieved when two columns in series were utilized. The (*R*)-enantiomer eluted first, with the retention

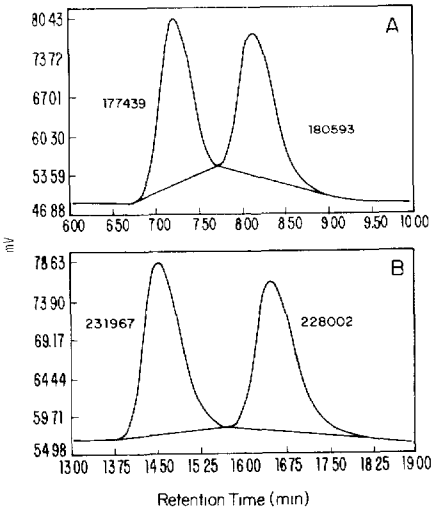


Fig. 2 Separation of the enantiomers of **1** on a chiral bovine serum albumin column. (A) One column (150 mm × 4 mm I.D., BSA-7, Resolvosil); (B) Two columns in series. Mobile phase, 2-propanol–0.025 *M* phosphate buffer (pH 7.6), flow-rate 0.5 ml/min; 100 ng of **1** injected in 10 µl mobile phase.

time (t_R) being ca. 14.5 min, followed by the (*S*)-enantiomer (t_R 16.5 min). The separation was not significantly affected by the volume of the sample injected on the column up to 200 µl. Several other chiral columns were explored (see Experimental), but either no separation of enantiomers was observed (EnantioPac, Pirkle, Chiralcel CA-1, Chiralcel WM) or separation was incomplete (β-cyclodextrin). A baseline separation was also observed on a Chiralpak OC column, but poor peak shapes precluded further utilization of this CSP for the assay development.

The effect of pH and the organic modifier (2-propanol) content in the mobile phase on the separation of enantiomers on BSA-7 columns has been studied. The optimum pH was found to be at 7.6, whereas at lower pH (6.2) and when the content of 2-propanol in mobile phase was increased from 1 to 2 or 4%, the two enantiomeric peaks were only partially resolved.

Direct stereoselective assay

Based on the separation of enantiomers on BSA-7 columns, an assay in whole blood has been developed in the concentration range 25–250 ng/ml of each enantiomer. The extraction of **1** from plasma was accomplished using the same procedure as described in the NSS assay (vide infra) except the acidic portion after back-extraction was neutralized with base to adjust solvent pH to that of the mobile phase (see Experimental). This adjustment was necessary to assure good resolution and acceptable peak shapes. The within-day precision (Table

TABLE I

INTRA-DAY PRECISION DATA FOR THE ANALYSIS OF ENANTIOMERS OF **1** IN WHOLE BLOOD

Concentration (ng/ml)	Coefficient of variation ($n=5$) (%)	
	(*R*)-**1**	(*S*)-**1**
25	5.2	5.4
50	4.8	4 6
125	3.6	3.5
250	4.1	3.9

I) of the assay was less than 6% for all concentrations within the standard curve range.

Peak areas of (*S*)- and (*R*)-enantiomers correlated linearly with concentrations of the isomers in the range of concentrations tested. The typical equation of the linear regression line was $y=328x+2040$ and $y=326x+916$ with the correlation coefficients of 0.99969 and 0.99971 for the (*R*)- and (*S*)-enantiomers, respectively. The accuracy of the assay expressed as (mean observed concentration)/(expected concentration) $\times 100$ was 98–102 and 97–103% for (*R*)-**1** and (*S*)-**1**. The combined recovery was $>80\%$ at all concentrations.

Non-stereoselective assays

Following the procedure for the NSS assay described in Experimental, the assays in whole blood, urine and plasma have been fully validated in the concentration range 2.5–100 ng/ml. The upper limit for the urine assay was 75 ng/ml. The within-day precision was less than 10% for all concentrations within the standard curve range (Table II).

TABLE II

INTRA-DAY VARIABILITY OF THE ASSAY OF **1** IN BIOLOGICAL FLUIDS

Concentration (ng/ml)	Coefficient of variation ($n=5$) (%)		
	Whole Blood	Plasma	Urine
2 5	6.6	4.7	4.2
5	6.7	6.4	6.5
10	4.9	2.9	4.9
25	0.5	2.4	4.3
50	2.8	3.0	4.0
100	2.0	2.8	1 0[a]

[a]The upper limit for the urine assay was 75 ng/ml.

TABLE III

INTER-DAY VARIABILITY FOR THE ASSAY OF QUALITY CONTROL WHOLE BLOOD, PLASMA AND URINE SAMPLES SPIKED WITH **1**

Sample	*n*	Nominal concentration (ng/ml)	Mean calculated concentration (ng/ml)	Coefficient of variation (%)
Whole blood	4	5	5.1	5.2
		50	48.9	9.6
Plasma	5	5	5.1	3.1
		50	50 3	4.8
Urine	2	5	5 0	0.8
		50	50 2	1.1

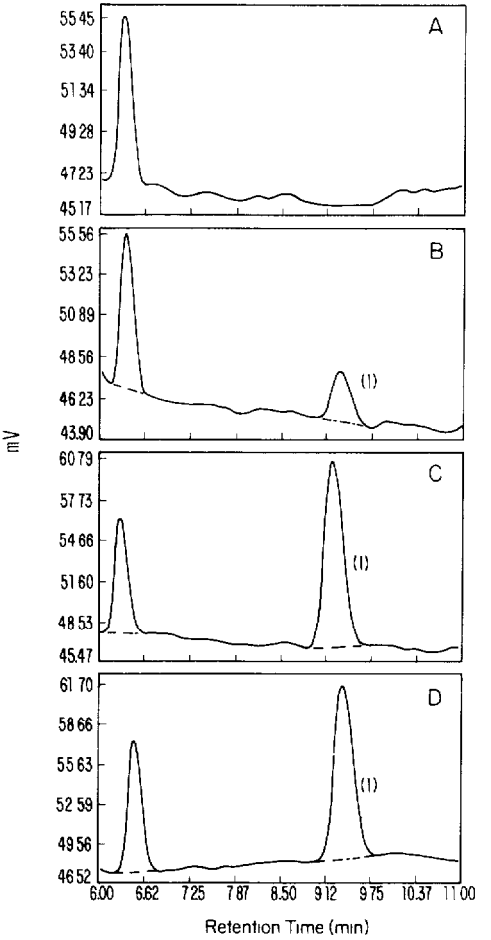


Fig. 3. Representative chromatograms of human whole blood samples spiked with **1**. (A) Blank whole blood; (B) whole blood spiked with 5 ng/ml **1**; (C) whole blood spiked with 25 ng/ml **1**, (D) subject whole blood 15 min after topical ocular administration of three drops of 2% ophthalmic solution to each eye in a 2-min interval (concentration of **1** equivalent to 24.2 ng/ml).

Inter-day precision, as measured by the concentration of QC standards, was also below 10% in all biological fluids analyzed (Table III). The accuracy of the assay based on QC standards at 5 and 50 ng/ml was 98–103, 101–102 and 100–101% in whole blood, plasma and urine, respectively. The correlation coefficients of the typical calibration curves were 0.9999, 0.9984 and 0.9996. Some representative chromatograms are presented in Fig. 3.

Analysis of samples from clinical studies

The representative concentration–time data of **1** in whole blood for selected subjects ($n=7$) after topical ocular administration of 2% ophthalmic solution is presented in Table IV. Each subject received a total of six drops of **1** administered as one drop in each eye repeated three times 1 min apart. Plasma and whole blood samples were collected for drug assay at 0 h (predose) and various times after the administration of the first dose up to fourteen days.

The corresponding plasma concentrations were all below the quantification limit of the assay (2.5 ng/ml). The representative concentrations of **1** in urine for selected subjects are given in Table V.

TABLE IV

WHOLE BLOOD LEVELS OF **1** AFTER BILATERAL TOPICAL OCULAR ADMINISTRATION OF A 2% OPHTHALMIC SOLUTION OF **1** TO HUMAN SUBJECTS

Six drops in total were administered The effective dose was estimated by determining the concentration of the drug in six drops of dosing solution from individual ocumeters used for dosing and subtracting the percentage of total dose recovered in facial tissues due to drug overflow and tearing which varied from 24 to 76%. The effective dose for subjects 1 and 2 was 0 78 and 1.11 mg, respectively.

Time (min)	Concentration (ng/ml)	
	Subject 1	Subject 2
0	0	0
5	13.2	25 6
30	29.0	58.7
90	41.4	71 6
120	55.9	74 3
240	45.6	80.6
360	42.0	80.5
1440	39.2	66.8
14 days	15.1	25.5

TABLE V

CONCENTRATIONS AND AMOUNTS OF **1** EXCRETED IN URINE AFTER BILATERAL TOPICAL OCULAR ADMINISTRATION OF A 2% OPHTHALMIC SOLUTION OF **1** TO SELECTED HUMAN SUBJECTS

Six drops in total were administered. For the effective dose, see Table IV.

Time (h)	Subject 1		Subject 2	
	Mean concentration (ng/ml)	Amount excreted[a] (μg)	Mean concentration (ng/ml)	Amount excreted[a] (μg)
Pre-dose	0	—	0	—
0- 4	35.6	6.6	54.3	7.3
4- 8	42.6	7.6	57.4	8.7
8-14	15.9	12 9	10.2	13 7
72	0.0	—	18.4	2.7
336	0.0	—	0.0	—

[a]Calculated by multiplying the volume (ml) of urine excreted in any given time interval by the concentration (ng/ml).

DISCUSSION

Various approaches to the direct chiral separation of enantiomers of **1** were evaluated; among them were separation on CSPs and utilization of chiral mobile phase additives with chiral chromatography. The latter approach using ZGP (3.5 m*M*) as chiral mobile phase (methylene chloride) additive and chromatography on an achiral diol column did not lead to the separation of enantiomers. In addition, since the absorption (A) of ZGP at the detection wavelength (252 nm) was rather significant ($A = 1.2$ in a 1-cm cell), a considerable UV detector baseline instability was encountered, preventing reliable chromatographic performance of such a system.

In the second general approach, six different CSPs were evaluated. The adequate separation and chromatography was achieved on a protein-bonded phase consisting of BSA attached to silica. Since the retention properties of protein columns are highly dependent on proper selection of mobile phase parameters, such as pH, ionic concentration and the concentration of organic modifier, all these parameters were optimized. Almost baseline separation was achieved when two columns in series were employed (Fig. 2). The BSA columns were used with buffered aqueous mobile phases with a low concentration of an organic modifier, which allowed direct injection of aqueous samples onto the column and facilitated analysis of biological samples. The chiral assay in whole blood based on this approach has been developed and fully validated in the 25–250 ng/ml concentration range of each enantiomer. The assay has been uti-

lized for the analyses of about 100 samples without any signs of loss in resolution or column performance.

Under isocratic conditions utilized in the assay and due to the low content of the organic modifier in the mobile phase (1% 2-propanol), there was a potential for the appearance of more hydrophobic impurity peaks from previous injections coeluting with drug peak in subsequent analyses. This was found not to be the case, however, probably due to an efficient elimination of these impurities in the back-extraction step utilized in the liquid–liquid extraction scheme. The hydrophobic impurities extractable from whole blood or plasma were effectively retained in the organic phase which was discarded.

After the full assay evaluation and analyses of about 100 whole blood samples, the chiral BSA columns were stored under the conditions recommended by the manufacturer. After a month of storage, the baseline resolution of the enantiomers was partially lost. Also, the resolution was dependent on the source of the column, its age, batch number, storage conditions, etc. For these reasons, and because of other limitations in the use of direct CSP methodology for multi-sample analyses of biological fluids [21,22], it was very unlikely that the direct chiral separation method will be rugged enough to support the full-scale human pharmacokinetic studies involving large numbers of samples. Therefore, in addition to this method, an indirect chiral assay based on derivatization with a homochiral reagent [(+)-(*S*)-(1-naphthyl)ethylisocyanate] has also been developed [20]. Some newer advances in the BSA immobilization technique [23,24] and column technology [25] could make the CSP approach applicable to large-scale analyses of clinical samples in the future.

Using the indirect stereoselective assay methodology, it was established that no in vivo conversion of the (*S*)- to (*R*)-isomer was occurring in humans. Therefore, the more sensitive (2.5 ng/ml) and more rugged NSS assay was developed to support future pharmacokinetic studies involving (*S*)-**1**. In addition to whole blood, the NSS assays in plasma and urine have also been developed.

The efficient, high-recovery liquid–liquid extraction of **1** from whole blood or plasma at concentrations below 100 ng/ml required detailed studies of the following steps: deproteinization, pH adjustment before drug extraction and back-extraction with an acid to avoid evaporation of a large amount of solvents. The NSS procedure in whole blood used in preclinical studies [26] was inadequate for multi-sample clinical analyses of **1** at concentrations below 100 ng/ml. The recovery of the drug at low concentrations was greatly improved when the deproteinization step with TCA was employed instead of the direct extraction from plasma. Among various solvents utilized, the combination of solvents used in the final assay (see Experimental) was found to give not only good recoveries but also gave samples free of impurities in the drug peak region of the chromatograms at low concentrations. Because of the possibility of proton removal from the sulfonamide group of **1** at high pH values (pK_a values of

6.0 and 8.5), the utilization of buffered (pH 8) instead of strongly basic solutions before extraction with the organic solvents also greatly improved recovery. Finally, the back-extraction of basic **1** from the organic phase with weak phosphoric acid instead of evaporation of solvent to dryness and reconstitution in the mobile phase also improved recovery and considerably speeded up the sample preparation time, making the assay capable of analyzing more than 50 clinical samples per day. The assays developed were fully applicable for multi-sample analyses and clinical pharmacokinetic evaluation of **1**.

The results of the pilot human pharmacokinetic studies with **1** indicated that appreciable drug levels appeared in RBCs shortly (5 min) after first drop of **1** was topically applied to an eye, reaching the maximum at about 2–4 h after dosing. Even after fourteen days after dosing, the appreciable concentrations of **1** in RBCs were detected (Table IV). Drug levels in plasma were much lower, below the quantification limit of the NSS assay (2.5 ng/ml). Only about 1% of the total dose was recovered unchanged in urine. The re-analysis of the same samples using the SS assay [20] indicated that **1** present in whole blood was almost exclusively in the form of the (*S*)-enantiomer and in urine predominantly in the form of the (*R*)-enantiomer. The full clinical pharmacokinetic evaluation of **1** and its enantiomers based on the NSS and SS analyses will be the subject of a separate paper.

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