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Determination of chiral sulfoxides in plasma by normal-phase liquid chromatography–atmospheric pressure chemical ionization mass spectrometry[☆]

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Abstract

A sensitive and specific assay has been developed and validated for the separation of a chiral sulfoxide drug candidate with simultaneous determination of the corresponding sulfide and sulfone in plasma by normal-phase LC–MS–MS. Separation was achieved on a Chiralpak AD (100×2.1 mm) column with a 2-propanol–hexane (80:20) mobile phase within 7 min. Aqueous mobile phase (2-propanol–10 m*M* ammonium acetate, 75:25) was added post-column prior to introduction into the heated nebulizer interface of a Sciex API 3plus mass spectrometer, to avoid the explosion hazard of hexane-containing mobile phases in the presence of a corona discharge. The linear range of this assay was 5–2500 ng/ml. The accuracy and precision of the chiral sulfoxides, the sulfide and the sulfone were within ±15% across the linear range. The limit of quantitation for each component was 5 ng/ml based on the extraction of 0.25 ml plasma. The recovery for each component was between 82 and 120%. This assay was sufficiently sensitive and specific to support pre-clinical development studies in rats, dogs and monkeys. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

A number of drugs on the market have been developed as racemic mixtures, where either one or both of the enantiomers are active. Oftentimes, enantiomers may exhibit distinct activities, toxicities, and pharmacokinetic and metabolic profiles. In recent years, chiral syntheses have become more commonplace and chiral preparatory separations more reasonable. Improvements in chiral separations using mobile phase additives and varying chiral stationary phases have made enantiomeric separations more routine. As a result of these synthetic and analytical improvements, it is possible to not only identify the more potent enantiomer, but also study the pharmacokinetic and metabolic profile of each enantiomer. Recognizing these advances, the US Food and Drug Administration (FDA) has suggested and pharmaceutical companies have opted to develop only one of the enantiomers, rather than embark in

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thorough studies for each of the species [1]. Likewise, development teams have recognized the utility of investigating the potential differences between enantiomers in the early stages of discovery. Consequently, analytical methodologies capable of separating enantiomers have become necessary in discovery screening laboratories.

Since it is now universally accepted that liquid chromatography–mass spectrometry (LC–MS) and LC–MS–MS are the tools of choice in a drug-discovery laboratory, it follows, from the increasing demands to employ chiral methods in screening environments, that the two techniques, LC–MS and chiral separations, would naturally be coupled. A number of investigators have illustrated the benefits of using LC–MS for improved sensitivity of chiral enantiomers [2–4]. And a previous report on the optimization of enantiomeric separations using LC–MS [5] indicated the possibility that methods already developed in the purification of pure enantiomers could be coupled to mass spectrometry in order to meet the demands of discovery screening. Merely coupling the two techniques is not enough. These chiral methods must also be sufficiently general to be readily used in the fast-paced screening environment.

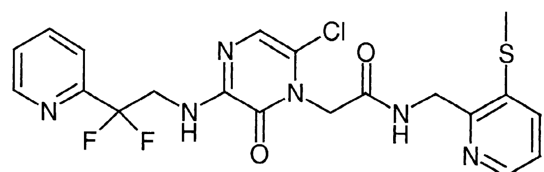
In one such screening effort at Merck, a chiral sulfoxide was identified as a promising drug candidate and prepared as separate enantiomers. Although both enantiomers were pharmacologically active, enantiomer 1 was more potent. During initial pharmacokinetic and metabolic characterizations of the sulfoxide, it was found that the sulfoxide was reduced and oxidized in vitro and in vivo to form the sulfide and the sulfone analogs, which were also active compounds. Additionally, the sulfide was itself metabolized to the sulfoxide, but as the initial studies were carried out using an achiral reversed-phase system, we were unable to ascertain whether this biotransformation gave rise to chiral inversion. Therefore, a chiral assay was required in order to observe both enantiomers when each was studied independently in vitro and in vivo, to evaluate the potential for interconversion of the sulfoxide to the sulfide and sulfone, and to determine the pharmacokinetic and metabolic properties of the individual enantiomers.

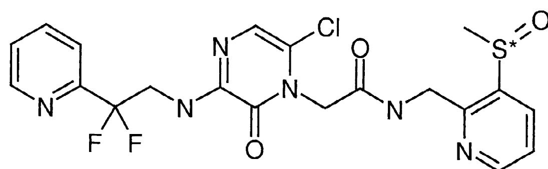
In recent years, a number of bioactive sulfoxides have been investigated using chiral methods to characterize the biological activities of the respective enantiomers [6–10]. These separations were achieved using a number of chiral stationary phases and ultraviolet or fluorescence detection [6–10]. In developing the method to characterize the in vitro and in vivo biological characteristics of the chiral sulfoxide identified from the screening effort at Merck, we coupled the chiral methodology used by the Department of Medicinal Chemistry in the preparation of the enantiomerically pure sulfoxides, to LC–MS–MS. The preparative-scale isolation employed a Chiralpak AD column and a hexane–isopropanol mobile phase. Unfortunately, hexane-containing mobile phases are potentially explosive in the presence the corona discharge in LC–atmospheric pressure chemical ionization (APCI) MS–MS. In order to overcome this problem, aqueous solvent was added post-column to the chiral LC effluent prior to introduction to the mass spectrometer [2]. Thus, a sensitive and specific assay was developed and validated for the separation of the chiral sulfoxides, and their sulfide and sulfone metabolites. Since this chiral sulfoxide was identified early in discovery, determination of the optical rotation of the enantiomers was not performed. Therefore, the early eluting enantiomer will be designated enantiomer 1 and the later eluting enantiomer will be designated enantiomer 2 in the following discussions.

2. Materials and methods

2.1. Reagents

The sulfide, chiral sulfoxides and sulfone (Fig. 1) were synthesized by Merck Research Laboratories (West Point, PA, USA), HPLC-grade methanol, acetonitrile, 2-propanol, hexane and ACS-grade formic acid, hydrochloric acid and ammonium acetate were obtained from Fisher Scientific (Pittsburgh, PA, USA). Ultra-high-purity nitrogen was obtained from Praxair (Danbury, CT, USA) and ultra-high-purity argon was obtained from West Point Supply (West Point, PA, USA). Water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).





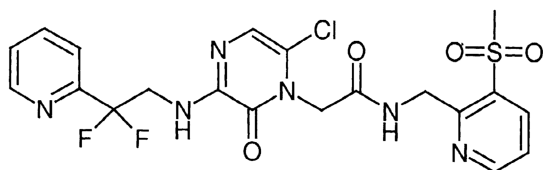


Fig. 1. Structures of the sulfide (top), chiral sulfoxide (middle) and sulfone (bottom). Chiral center is denoted by an asterisk.

2.2. Standard solutions

Stock standard solutions of the sulfide, chiral sulfoxides, sulfone and internal standard were prepared by dissolving appropriate amounts of the compounds in acetonitrile–methanol (50:50, v/v) containing 0.1% formic acid to give final free base concentrations of 1 mg/ml. Combined working standard solutions containing the sulfide, chiral sulfoxides and the sulfone at concentrations of 25, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 μg/ml were prepared daily by appropriate dilution of 1 mg/ml stocks with 20% acetonitrile containing 0.1% formic acid. Working internal standard solution was prepared by appropriate dilution of the internal standard stock with 20% acetonitrile containing 0.1% formic acid to give a solution concentration of 400 ng/ml.

2.3. Quality control solutions

Quality control stock solutions of the sulfide, chiral sulfoxides and sulfone were prepared, using separate weighings, by dissolving appropriate amounts of the compounds in acetonitrile–methanol (50:50, v/v) containing 0.1% formic acid to give final free base concentrations of 1 mg/ml. Combined quality control working solutions containing the sulfide, chiral sulfoxides and the sulfone at concentrations of 10, 2, 0.1 and 0.05 μg/ml were prepared daily by appropriate dilution of the 1 mg/ml quality control stocks with 20% acetonitrile containing 0.1% formic acid.

2.4. Sample preparation and extraction

Control plasma was centrifuged for 10 min at 3000 rpm prior to aliquoting. Aliquots of control plasma (0.25 ml) were pipetted into 75×12 mm glass tubes and 25 μl of appropriate combined standard or quality control working solutions was added to yield nominal concentrations of 5, 10, 20, 50, 100, 200, 500, 1000 and 2500 ng/ml for standards and 5, 10, 200 and 1000 ng/ml for quality control samples. Working internal standard solution (50 μl) was added to all samples and then the samples were vortex mixed. Hydrochloric acid (0.1 *M*, 0.25 ml) was added to all samples and the samples were vortex mixed. Solid-phase extraction was accomplished using a Quadra 96 workstation (Tomtec, Hawden, CT, USA). The Quadra 96 completed the following steps. (1) Conditioned a 96-well Oasis HLB 30 mg plate (Waters, Milford, MA, USA) by washing with 0.7 ml of methanol and 0.7 ml of water. Samples were manually transferred onto conditioned cartridges without vacuum applied and drawn slowly through the column with low vacuum. (2) Washed columns with 0.7 ml of water and 0.7 ml of 5% methanol in water. (3) Eluted analytes and internal standard with 0.7 ml of 70% acetonitrile in water. The plate was remove from the Tomtec deck and the eluate evaporated to dryness in an Evaparray (Jones Chromatography, Lakewood, CO, USA) 96-well sample concentrator at 55 °C. Residue was

reconstituted with 150 μl of 2-propanol–hexane (75:25). Samples were vortex mixed for 1 min and sonicated in an ultrasonic agitator for 10 min. Samples were transferred to inserts in 96-well round bottom plates and inserts were capped with snap caps. The plate was centrifuged for 10 min at 4000 rpm.

2.5. High-performance liquid chromatography

The liquid chromatograph consisted of a Hewlett-Packard 1050 quaternary pump (Palo Alto, CA, USA), an Alltech 426 HPLC pump (Deerfield, IL, USA) and a HTS PAL Autosampler (Leap Technologies, Carrboro, NC, USA) interfaced via Sciex's heated nebulizer (APCI) to a Sciex API IIIplus triple quadrupole mass spectrometer (Perkin-Elmer/Sciex, Thornhill, Canada). Chromatographic separations were carried out on a ChiralPak AD 10 μm, 10 cm×2.1 mm I.D. column (Chiral Technologies, Exton, PA, USA). The isocratic mobile phase consisted of 2-propanol–hexane (80:20, v/v) pumped at 0.25 ml/min. Isopropanol–10 m*M* ammonium acetate in water (75:25, v/v) was added post-column to the eluate at a flow-rate of 0.75 ml/min prior to splitless introduction into the APCI interface of the mass spectrometer. The chromatographic run time was 7 min.

2.6. Mass spectrometric parameters

The Sciex API IIIplus triple quadrupole mass spectrometer was operated in positive ionization selected reaction monitoring (SRM) mode using a heated nebulizer (APCI) interface with a discharge needle current (DI) of 4 μA. The nebulization gas (nitrogen) was 80 p.s.i. and the auxiliary and curtain gas flows (both nitrogen) were 2.0 and 1.2 l/min, respectively (1 p.s.i.=6894.76 Pa). The orifice potential was set at 45 V. The heated nebulizer temperature was set at 500 °C and the sample pump was operated with a source delta value of 0.8 in of water. Ions were collisionally activated at an energy of 25 eV and the collision gas (argon) thickness was $300 \cdot 10^{13}$ molecules/cm^2. This assay was based on monitoring the pseudomolecular $[M+H]^+$ ions for the sulfide, chiral sulfoxides, sulfone and internal standard in the first quadrupole and their corresponding product fragments in the third quadrupole. The dwell time for each ion was 200 ms. SRM chromatographic data were collected using Sciex RAD software. Calibration curves were prepared using Sciex Mac Quan software by plotting peak area ratios of analyte to internal standard against concentrations of each analyte using a linear regression weighting of $1/X^2$ where X is plasma concentration. Concentrations were determined by interpolation from the appropriate standard curve.

2.7. Validation procedures

The analytical method was validated in dog plasma using five standard curves and 10 replicate quality controls at four concentrations containing the sulfide, chiral sulfoxides and the sulfone. Standard curves were prepared by adding appropriate volumes of standard solutions to plasma (0.25 ml) to yield concentrations in the range 5–2500 ng/ml. Quality control samples were prepared by adding appropriate volumes of quality control solutions to plasma (0.25 ml) to yield concentrations of 5, 10, 200 and 1000 ng/ml. Standard curves were constructed using a weighted ($1/X^2$) linear regression. Inter-day precision and accuracy were determined using duplicate quality control results back-calculated from each of the five curves. Intra-day precision and accuracy were determined using the readback values of five sets of standards from one of the standard curves.

3. Results and discussion

In our discovery screening, it was necessary to develop a sensitive and specific chiral assay for a potent sulfoxide drug candidate and its corresponding sulfide and sulfone metabolites to address issues of reversible metabolism and chiral inversion. In order to support early drug metabolism studies for compounds identified in discovery screening, rapid method development is necessary. To minimize method development time, chromatography conditions established in medicinal chemistry were adapted for use with mass spectrometry. The mass spectrometer would provide the sensitivity needed to support in-vivo studies and also differentiate between the co-eluting sulfide and sulfone metabolites. Nor-

mal-phase LC–MS–MS was not typically used for analysis in our laboratory due to safety concerns, but post-column reagent addition solved this problem.

3.1. Interfacing normal-phase solvents into heated nebulizer interface of mass spectrometer

Hexane-containing mobile phases in the presence of a corona discharge are potentially explosive. Post-column addition of aqueous ammonium acetate (10 m*M*) and isopropanol to the mobile phase eluate prior to introduction into the heated nebulizer provided a means to overcome the safety and miscibility issues associated with hexane-containing mobile phases [3]. A make-up solvent of 2-propanol–10 m*M* ammonium acetate (75:25) at a flow-rate of 0.75 ml/min was added to the post-column mobile phase eluate (0.25 ml/min). This combined solvent system eliminated any explosion hazard through the introduction of water into the interface. The isopropanol present reduced immiscibility with hexane, and the addition of ammonium acetate aided the ionization process.

3.2. Recovery

Absolute recovery was determined at 5 and 1000 ng/ml. When using the chiral assay, recovery at 5 ng/ml ranged from 118 to 131% for all the compounds analyzed. Recovery at 1000 ng/ml ranged from 87 to 95% for all compounds analyzed. This recovery study was repeated with similar results. These recovery results indicate that there may be more variability reconstituting extracts in normal-phase solvents at lower concentrations than in reverse phase solvents. To confirm this, recovery studies were then repeated using the same sample preparation and extraction procedures described here, and analyzed by reversed-phase chromatography. The two chiral sulfoxides were prepared and analyzed separately since the reversed-phase system could not differentiate between the enantiomers. Plasma standards were prepared in triplicate at 5 and 1000 ng/ml along with blank plasma. Following solid-phase extraction, blank plasma extracts were spiked with standards (in triplicate) and used as recovery standards. All samples were evaporated, reconstituted and analyzed by achiral LC–MS–MS. The ratio of the mean peak area for standards over the mean peak area for recovery standards was used to determine recovery. Using this procedure, the recovery at 1000 ng/ml was 82–120% for all compounds and 96–106% at 5 ng/ml for all compounds. The recovery for the internal standard was 86%.

3.3. Sensitivity, linearity and selectivity

The limit of quantitation (LOQ) based on a 0.25 ml plasma aliquot was 5 ng/ml for all components. The LOQ was determined from validation data by calculating the relative standard deviation (RSD) of back-calculated standard curve concentrations from five curves read-back from one of the standard curves (intra-day precision). The RSDs for all compounds were $\leq$15% at 5 ng/ml (Table 1). A linear response for peak area ratios of analyte to internal standard was observed for concentration from 5 to 2500 ng/ml for the sulfide, chiral sulfoxides and the sulfone. Using a weighted $(1/X^2)$ linear regression, correlation coefficients were typically 0.997–1.000. SRM chromatograms of blank plasma showed no interference from endogenous plasma components (Fig. 2).

3.4. Accuracy and precision

Inter-day precision for the standards and quality control samples, determined using the read-back values of each curve and its duplicate set of quality control samples treated independently, are shown in Table 2. Intra-day precision, determined using read-back values of five sets of standards from one standard curve are show in Table 1. The mean accuracy, indicated by the ratio of actual to theoretical concentrations, is also shown in Tables 1 and 2.

3.5. Assay applicability

This validated assay was used to support drug metabolism studies following intravenous (i.v.) and oral administration of the two enantiomers separately, and also administration of the sulfide and sulfone to rats, dogs and monkeys. Analysis of these studies indicated that the sulfone was present as a major metabolite in all animal species, and bio-

Table 1
Intra-day accuracy and precision data for the sulfide, enantiomer 1, enantiomer 2 and the sulfone in dog plasma

	Standard curve samples								
	5 ng/ml	10 ng/ml	20 ng/ml	50 ng/ml	100 ng/ml	200 ng/ml	500 ng/ml	1000 ng/ml	2500 ng/ml
Enantiomer 1									
Mean	4.86	10.40	19.26	49.72	95.85	182.10	461.43	883.89	2355.46
SD	0.37	1.04	1.61	3.10	8.99	20.28	47.88	75.13	291.92
RSD (%)	7.58	10.02	8.37	6.23	9.37	11.14	10.38	8.50	12.39
Accuracy	97.28	104.00	96.30	99.44	95.85	91.05	92.29	88.39	94.22
Enantiomer 2									
Mean	5.22	10.84	20.69	53.91	102.44	197.55	510.22	981.36	2600.74
SD	0.60	1.00	1.46	3.92	9.57	19.29	54.83	83.43	311.88
RSD (%)	11.51	9.24	7.06	7.27	9.34	9.76	10.75	8.50	11.99
Accuracy	104.32	108.42	103.44	107.82	102.44	98.78	102.04	98.14	104.03
Sulfide									
Mean	5.28	10.35	20.62	53.22	102.50	200.50	496.70	967.63	2506.90
SD	0.80	1.00	1.85	1.71	9.43	16.07	53.56	86.82	264.59
RSD (%)	15.24	9.66	8.99	3.21	9.20	8.02	10.78	8.97	10.55
Accuracy	105.60	103.54	103.10	106.43	102.50	100.25	99.34	96.76	100.28
Sulfone									
Mean	4.95	10.39	20.36	52.52	104.01	204.57	515.29	977.68	2573.27
SD	0.55	0.95	2.02	3.62	9.62	21.47	62.39	99.42	339.03
RSD (%)	11.02	9.18	9.93	6.88	9.25	10.49	12.11	10.17	13.17
Accuracy	99.00	103.90	101.81	105.03	104.01	102.29	103.06	97.77	102.93

transformation to the sulfide was minimal. In addition, little to no chiral inversion was noted following administration of either sulfoxide isomer. The SRM chromatogram for an authentic dog plasma sample following i.v. administration of the more potent sulfoxide is shown in Fig. 3.

4. Conclusions

A normal-phase chiral LC–MS–MS assay was developed and validated to support early drug metabolism studies with Merck synthesized chiral sulfoxides, and the corresponding sulfide and sulfone

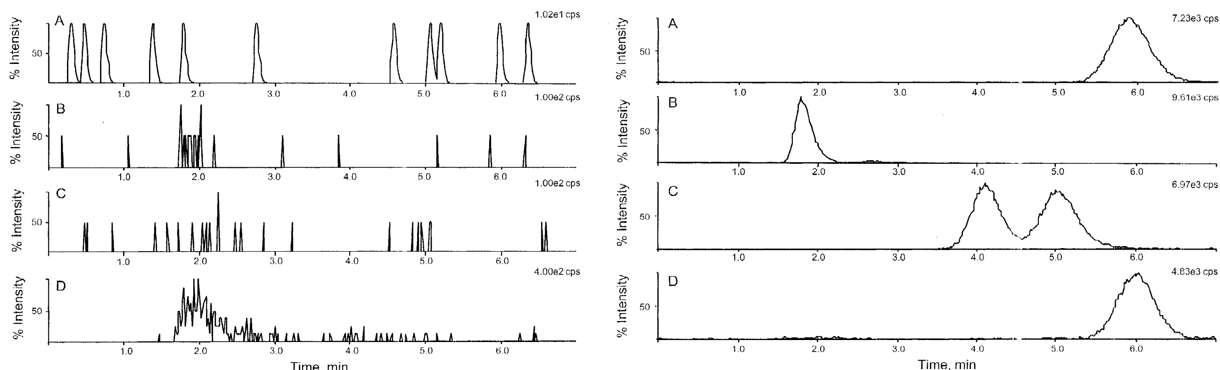


Fig. 2. Selected reaction monitoring chromatograms from a blank dog plasma extract (left) and a 100 ng/ml spiked standard extract. Sulfone (A), internal standard (B), enantiomer 1 (C, first peak), enantiomer 2 (C, second peak) and the sulfide (D).

Table 2
Inter-day accuracy and precision data for the sulfide, enantiomer 1, enantiomer 2, and the sulfone in dog plasma

	Standard curve samples									Quality control samples			
ng/mL	5	10	20	50	100	200	500	1000	2500	5	10	200	1000
Enantiomer 1													
Mean	4.82	10.63	20.07	52.50	101.15	192.29	487.90	970.32	2488.28	5.59	10.81	194.38	993.94
SD	0.09	0.49	1.05	3.99	3.91	7.28	10.28	18.19	84.05	0.58	0.96	7.49	18.38
RSD (%)	1.91	4.62	5.22	7.60	3.86	3.79	2.11	1.87	3.38	10.33	8.86	3.85	1.85
Accuracy	96.32	106.30	100.33	104.99	101.15	96.15	97.58	97.03	99.53	111.87	108.06	97.19	99.39
Enantiomer 2													
Mean	4.89	10.37	19.97	52.37	99.44	191.78	495.18	982.56	2522.34	5.28	10.43	195.78	1001.35
SD	0.19	0.68	0.93	3.61	4.23	5.41	14.82	22.52	76.93	0.51	0.62	7.01	34.92
RSD (%)	3.82	6.54	4.65	6.89	4.25	2.82	2.99	2.29	3.05	9.74	5.96	3.58	3.49
Accuracy	97.88	103.66	99.85	104.74	99.44	95.89	99.04	98.26	100.89	105.58	104.26	97.89	100.13
Sulfide													
Mean	4.97	10.00	20.12	52.51	101.03	197.87	489.57	978.23	2472.18	5.11	10.08	187.52	955.62
SD	0.16	0.59	0.43	3.03	5.17	6.02	20.57	31.39	85.71	0.55	0.62	9.73	46.26
RSD (%)	3.29	5.90	2.14	5.77	5.12	3.04	4.20	3.21	3.47	10.80	6.17	5.19	4.84
Accuracy	99.36	99.96	100.62	105.01	101.03	98.94	97.91	97.82	98.89	102.18	100.78	93.76	95.56
Sulfone													
Mean	4.94	10.22	19.88	51.11	100.93	198.18	498.14	979.19	2484.49	4.82	9.58	188.09	952.95
SD	0.13	0.42	0.59	1.91	3.89	6.90	16.30	23.64	82.51	0.63	0.67	6.16	22.19
RSD (%)	2.64	4.08	2.98	3.74	3.85	3.48	3.27	2.41	3.32	13.18	6.95	3.27	2.33
Accuracy	98.80	102.20	99.38	102.22	100.93	99.09	99.63	97.92	99.38	96.36	95.80	94.04	95.30

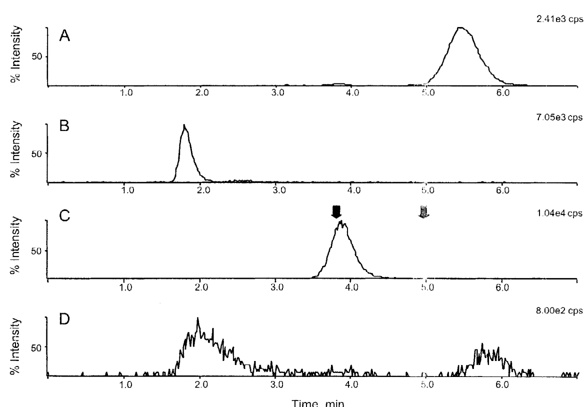


Fig. 3. Selected reaction monitoring chromatograms for an authentic dog plasma sample following i.v. administration of 1 mg/kg enantiomer 1. Sulfone (A) 168 ng/ml, internal standard (B) 80 ng/ml, enantiomer 1 (C, t_R=3.9 min) 820 ng/ml, enantiomer 2 (C, t_R=5 min) below LOQ, sulfide (D) 30 ng/ml.

metabolites. Separation of the sulfoxides was accomplished within 7 min using a Chiralpak AD 10 cm×2.1 mm chromatographic column. Post-column addition of isopropanol and ammonium acetate in water to the mobile phase eluate prior to entering the heated nebulizer of the mass spectrometer minimized the potential explosion hazard of the hexane-containing mobile phase, while maintaining miscibility of the two solutions. The linear range for each component in the assay was 5–2500 ng/ml with an LOQ of 5 ng/ml. This assay was sufficiently sensitive to quantitate levels of sulfoxide isomers, sulfide and sulfone in rat, dog and monkey plasma following intravenous and oral administration of each compound independently.

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References

[1] FDA Policy statement, Chirality 4 (1992) 338.
[2] T. Alebic-Kolbah, A.P. Zavitsanos, J. Chromatogr. A 759 (1997) 65.
[3] C. Rustichelli, V. Ferioli, G. Gamberini, Chromatographia 44 (1997).
[4] H. Stenhoff, A. Blomqvist, P.-O. Lagerstrom, J. Chromatogr. B 734 (1999) 191.
[5] S.A. Wood, J.M. Long, R.J. Simmonds, J.W. Bridges, D. Stevenson, J. Pharm. Biomed. Anal. 16 (1997) 231.
[6] M. Lienne, M. Caude, R. Rosset, A. Tambute, P. Delatour, Chirality 1 (1989) 142.
[7] M. Lienne, M. Caude, R. Rosset, J. Chromatogr. 472 (1989) 265.
[8] B.T. Butler, G. Silvey, D.M. Houston, D.R. Borcherding, V.L. Vaughn, A.T. McPhail, D.M. Radzik, H. Wynberg, W. Ten Hoeve, E. Van Echten, N.K. Ahmed, M.D. Linnik, Chirality 4 (1992) 155.
[9] C.B. Eap, L. Koeb, K. Powell, P. Baumann, J. Chromatogr. B 669 (1995) 271.
[10] F.O. Paias, V.L. Lanchote, O.M. Takayanagui, P.S. Bonato, Chirality 9 (1997) 722.