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Determination of MK-507, a novel topically effective carbonic anhydrase inhibitor, and its de-ethylated metabolite in human whole blood, plasma, and urine by high-performance liquid chromatography

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Abstract

Sensitive methods for the determination of a novel topically effective carbonic anhydrase inhibitor (CAI) I, MK-507, and its de-ethylated metabolite II, in human whole blood, plasma and urine were developed. These methods were based on liquid–liquid extraction of I and II from biological matrices, back extraction into acid, and analysis by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (252 nm). The assays were fully validated in the concentration range of 5 to 500 ng/ml, and the limit of quantification (LOQ) for I and II, defined as the lowest concentration on the standard curve for which precision (coefficient of variation, C.V.) is $< 10\%$, was 5 ng/ml in whole blood, plasma, and urine. These methods were applied for the analyses of biological fluid samples from a variety of clinical pharmacokinetic studies. In addition, a method in whole blood based on column-switching HPLC with UV detection and with an LOQ of 50 ng/ml was also developed. The switching valve was used to eliminate interferences from late eluting peaks extracted from whole blood. The details of these assays, together with some representative data from a human study, are presented.

1. Introduction

A number of topically effective carbonic anhydrase inhibitors possessing a thienothiopyran–2-sulfonamide moiety in the molecule have been identified recently as exhibiting good ocular hypotensive activity in various animal models and in humans [1]. Compound I (5,6-dihydro-(*S*)-4-(ethylamino)-(*S*)-6-methyl-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide, MK-507, Fig. 1) is a new member of this class of CAIs and is being evaluated as a potential antiglaucoma agent [2]. In order to support the clinical pharmacokinetic program, sensitive assays for determination of I and its de-ethylated metabolite II in biological fluids at low ng/ml levels were needed. All clinical studies were performed using the single stereoisomer with the *SS* absolute configuration around the two chiral centers (Fig. 1).

Initially, in order to assess the potential for the *in vivo* inversion of configuration at one or both chiral centers of I, a method for the chiral

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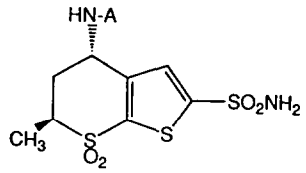

I; A = C_2H_5

II; A = H

Fig. 1. Chemical structures of I and its de-ethylated metabolite II.

separation of four stereoisomers of I was developed [3]. It was established, using this method, that no inversion of configuration was occuring, and only the *SS* isomer was present in the post-dose whole blood and urine samples from human subjects. In addition, in order to establish the absolute stereochemistry of the de-ethylated metabolite II, a different stereoselective method capable of separation of the four stereoisomers of II was developed [4]. This method was based on chiral derivatization and high-pressure liquid chromatography (HPLC) under normal-phase conditions. Again, a single major peak corresponding to isomer II was identified, confirming that the absolute stereochemistry of the metabolite was also *SS*. Based on all these data and lack of an *in vivo* inversion of the configuration around the two chiral centers in molecule I, non-stereoselective, sensitive assays were developed to support human pharmacokinetic studies involving compound I. The development of these assays in human whole blood, plasma, and urine with a limit of reliable quantification (LOQ) of 5 ng/ml is the subject of this paper. The assays were based on HPLC with ultraviolet (UV) detection and were modified during the course of the clinical program to include a column switching technique for the removal of late eluting endogenous impurities from biological fluids. The assays were successfully utilized for the analyses of large numbers of samples from a variety of human pharmacokinetic studies, and assay performance was assessed over an extended period of time.

2. Experimental

2.1. Materials

All solvents and reagents were of HPLC or analytical grade (Fisher, Fair Lawn, NJ, USA; Sigma, St. Louis, MO, USA). The drug-free human whole blood and plasma (heparinized) were purchased either from Biological Specialty (Lansdale, PA, USA) or Sera-Tec Biologicals (Harrisburg, PA, USA). Ion-pairing reagents, 1-nonanesulfonic acid sodium salt and octanesulfonic acid, originated from Lancaster Synthesis (Windham, NH, USA) and Aldrich (Milwaukee, WI, USA), respectively. The hydrochloride salt of I and maleate salt of II were synthesized in Merck Research Laboratories (West Point, PA, USA).

2.2. Instrumentation

A Waters Associates 703 HPLC system equipped with a 720 system controller, a WISP 715 autoinjector and a 6000A chromatographic pump (Waters-Millipore, Milford, MA, USA) were used for all analyses. When the column switching system was utilized, a Perkin-Elmer (Norwalk, CT, USA) Model 410 HPLC pump was also used. As a detector, an Applied Biosystems (Foster City, CA, USA) 785 variable-wavelength UV detector was employed. The detector output signal was interfaced either to a Hewlett-Packard laboratory automation system (HP 3357 LAS, Palo Alto, CA, USA) or to a Perkin-Elmer Nelson (Cupertino, CA, USA) Access-Chrom data system, via a PE-Nelson 900 series interface, for data collection, peak integration, and analyses.

In both non-column switching (NCS) and column switching (CS) versions of the assay, two chromatographic columns, a Beckman RP-8 (250 × 4.6 mm I.D., 5 μm, Rainin Instrument Co., Woburn, MA, USA, column 1) and an Analytichem RP-18 (50 × 4.6 mm I.D., 3 μm, Analytichem Int., Harbor City, CA, USA, column 2) in series were utilized. A Brownlee Labs (Santa Clara, CA, USA) RP-18 (25 × 4.6 mm I.D., 5 μm) column was utilized as a guard

column. For optimum performance, the guard column was replaced after 100 injections of biological fluid extracts. The columns were operated at ambient temperature (approximately 22°C). An electrically or pneumatically actuated six port column switching valve (Valco EC10W, or Rheodyne 7000) was used in the CS version of the assay.

2.3. Chromatographic conditions

The mobile phase in the NCS assay consisted of mixture of acetonitrile (500 ml) and a solution containing 2.4 g of the sodium salt of octanesulfonic acid in 0.085% phosphoric acid in water (1500 ml), delivered at a flow-rate of 1 ml/min. Prior to use, the mobile phase was passed through a 0.20-μm nylon membrane filter. In the urine assay, the volume ratio of acetonitrile to the ion-pairing part of the mobile phase was changed from 25:75 (v/v) to 24:76 (v/v) which was required to separate interfering peaks present in urine extracts. Under these conditions, retention times of 17.5 and 21.0 min for I and II, respectively, were observed. The autoinjector had a 28 min run time and the injection volume was 200 μl. The detector was set at $\lambda_{det} = 252$ nm, with a rise time of 2 s and absorbance setting of 0.002 AUFS.

In the CS version of the assay for whole blood, the mobile phase consisted of a mixture of 1500 ml of 0.085% phosphoric acid containing 2.53 g of the sodium salt of 1-nonanesulfonic acid and 560 ml acetonitrile. The same mobile phase was delivered by pumps 1 and 2 (see below) but at two different flow-rates of 1 and 2 ml/min, respectively.

2.4. Switching valve programming in the column switching assay

The block diagram of the HPLC system used in the CS version of the assay was similar to the system utilized by us earlier [5]. The times at which the events on pump 1 were set to trigger the pneumatic valve were determined daily by placing a piece of 0.007-in. I.D. tubing in place of column 2. While the valve was in position 1, 10 μl of 100 μg/ml working standard solution of I was injected and the time (t) at which the signal corresponding to peak I returned to the base-line level was determined (approximately $t = 4$ min). Since the retention time of metabolite II was shorter than that of I, both compounds eluted from column 1 into column 2 in time t. The timed events were then programmed to switch the valve to position 1 at the beginning of the run, switch the valve to position 2 at time t, and then switch it back to position 1 at 20 min after injection. The total run time, injection volume, and the detector absorbance setting were 20 min, 50 μl, and 0.05 AUFS, respectively.

2.5. Standard solutions

A stock solution of parent compound and metabolite (1 mg/ml) was prepared in methanol. This solution was further diluted to give a series of working standards with concentrations of 5.0, 2.0, 1.5, 1.0, 0.5, 0.2, 0.1, and 0.05 μg/ml. The stock standard was prepared once a month, and working standards were prepared weekly. All standards were stored at 5°C.

In the CS version of the assay, a series of working standards with concentrations of 100, 80, 40, and 20 μg/ml was prepared from a stock solution of 200 μg/ml of both I and II. Similar working standards with concentrations of 10, 4, 2, and 1 μg/ml were prepared from a separate stock solution (20 μg/ml) of I and II.

In addition, a series of quality control (QC) samples at two different concentrations were prepared by transferring a 200-μl aliquot of an appropriate and separately prepared standard solution containing I and II to a 50-ml volumetric flask and diluting to mark with either whole blood, plasma, and urine. The 1.25-ml aliquots of these solutions were placed in 2-ml plastic tubes, stored at −20°C, and analyzed daily with the study samples.

2.6. Sample preparation

Whole blood and plasma

A 1-ml aliquot of whole blood was pipetted into a 30-ml conical polypropylene centrifuge

tube and a 600-μl aliquot of 10% trichloroacetic acid (TCAA) was added to precipitate proteins. The content of the tube was vortex-mixed vigorously for 60 s, and 7 ml of 0.2 *M* phosphate buffer (pH 8) was added. The mixture was vortex-mixed again followed by the addition of 10 ml of an extraction solvent composed of toluene–ethyl acetate–2-propanol (40:50:10, v/v/v). After shaking the mixture for 20 min on a flat bed shaker, the tubes were centrifuged at 3000 rpm (2060 *g*) for 5 min, and 8 ml of the upper organic phase was transferred to a clean 15-ml polypropylene conical tube containing 300 μl of 0.015 *M* phosphoric acid. The tubes were shaken for 20 min at 60 shakes/min, centrifuged, the organic layer was aspirated to waste, and 200 μl (NCS assays) or 50 μl (CS whole blood assay) of the aqueous phase was injected onto the HPLC system.

Urine

A 1-ml aliquot of urine was placed in a 30-ml polypropylene tube, containing 1 ml of 0.2 *M* phosphate buffer (pH 8). After vortex-mixing, 10 ml of the extraction solvent (see above) was added. The extraction steps were similar to those used during the assay of whole blood and plasma. A 150-μl aliquot of the aqueous phase was injected onto the HPLC system.

2.7. Precision, accuracy, recovery, and specificity

The precision of the method was determined by replicate analyses ($n = 5$) of human whole blood, plasma, and urine containing I and II at all concentrations utilized for constructing calibration curves. The linearity of each standard line was confirmed by plotting the peak area (NCS assay) or peak height (CS assay) of drug or metabolite *versus* concentration. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted ($1/y$) linear regression of the standard line. The accuracy of the assay was expressed by (mean observed concentration)/(spiked concentration) × 100.

Assay selectivity was assessed by running blank control and patients' pre-dose biological fluid samples. No endogenous interferences were observed. The recovery was determined by comparing the peak area of I and II extracted from a biological fluid to that of standards injected directly.

3. Results

3.1. Whole blood, plasma, and urine assay

Following the procedure described in the Experimental section, the NCS assays in whole blood, plasma and urine were fully validated in the concentration range 5–500 ng/ml. The within-day precision expressed as the coefficient of variation (C.V., %) was less than 10% at all concentrations (Table 1). The accuracy for both I and II in all biological fluids and at all points within the standard curve range was 94–107%.

Inter-day variability, as measured by the concentration of QC standards, was also $< 10\%$ (Table 2).

In addition, analyses of QC samples (Table 2) indicated that both I and II were stable in whole blood at −20°C for a period of up to seven months.

Recovery of I was $75 \pm 4\%$, $70 \pm 5\%$, and $75 \pm 12\%$ from whole blood, plasma and urine, respectively, and was practically the same at all concentrations within the standard curve range. Recovery of the metabolite II from urine [$70 \pm 6\%$] was comparable to that of the parent compound, but was much lower from whole blood [$32 \pm 1\%$] and plasma [$35 \pm 4\%$]. In spite of this relatively low recovery observed in the latter two cases, the recovery was constant over the whole concentration range studied permitting reliable quantification of II.

Typical equations for the calibration line were $y = 860x - 2500$ and $y = 317x - 770$ for I and II in whole blood (with correlation coefficients of 0.9992 and 0.9995), $y = 915x - 710$ and $y = 391x - 170$ in plasma (with correlation coefficients of 0.9995 and 0.9998), and $y = 536x + 370$ and $y = 464x - 240$ in urine (with correlation coefficients of 0.9998 and 0.9999 for I and II,

Table 1
Intra-day precision and accuracy of the NCS assay of I and II in human whole blood, plasma and urine

Spiked concentration (ng/ml)	Whole blood		Plasma		Urine	
	C.V.[a] (%)	Accuracy[b] (%)	C.V.[a] (%)	Accuracy[b] (%)	C.V.[a] (%)	Accuracy[b] (%)
Parent drug (I)						
5.0	5.0	96	5.5	94	7.1	94
10.0	4.4	99	1.5	102	6.3	107
20.0	2.4	100	1.5	98	2.6	102
50.0	2.8	101	2.2	99	0.6	103
100.0	0.5	100	1.1	101	1.5	102
150.0	0.4	100	0.8	100	0.3	102
200.0	0.7	97	1.2	102	1.1	100
500.0	1.2	102	0.3	100	1.2	98
Metabolite (II)						
5.0	2.2	104	4.1	94	5.3	98
10.0	4.7	96	8.6	106	3.7	99
20.0	4.8	102	5.5	104	2.1	103
50.0	3.8	101	1.8	102	2.5	102
100.0	0.7	100	1.3	100	0.6	101
150.0	0.8	99	0.8	99	1.1	103
200.0	0.8	97	0.8	101	0.9	101
500.0	1.6	102	0.8	99	1.0	98

[a]Coefficient of variation ($n = 5$).
[b]Expressed as (mean observed concentrations)/(spiked concentration) × 100 ($n = 5$).

Table 2
Inter-day variability for the NCS assay of quality control samples spiked with I and II

Biological specimen	Spiked concentration (ng/ml)	Number of determinations	Mean calculated concentrations (ng/ml)		C.V. (%)	
			I	II	I	II
Blood	20.0	20[a]	19.9	20.1	7.8	7.0
	400.0	20[a]	405.0	404.6	4.2	4.7
Plasma	20.0	11[b]	20.5	19.4	10.0	8.1
	400.0	11[b]	397.8	400.2	4.9	5.5
Urine	20.0	8[c]	21.2	20.7	7.6	4.7
	400.0	8[c]	402.2	400.6	3.9	3.8

[a]Over a period of 202 days.
[b]Over a period of 84 days.
[c]Over a period of 98 days.

respectively). Representative chromatograms are presented in Fig. 2.

A late eluting peak with a retention time (t_R) of *ca.* 115–120 min was observed in whole blood and plasma extracts. In order to eliminate any interference of this peak with the peaks of I and II in subsequent injections, the analysis time between injections was adjusted in such a way as to place the late eluting peak in the solvent front of the next sample injected. In the CS assay, a

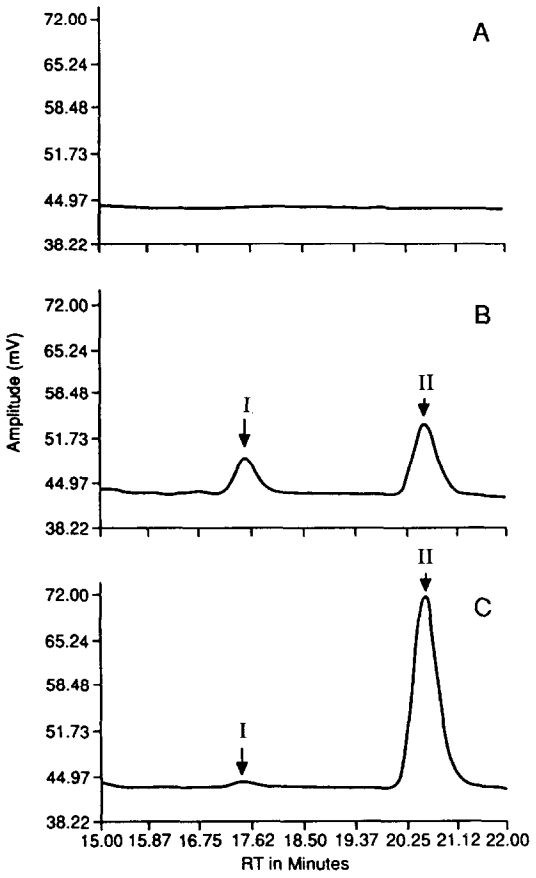

Fig. 2. Expanded chromatograms (15–22 min) of human whole blood samples spiked with I and II using NCS assay. (A) Blank control blood; (B) Whole blood spiked with 100 ng/ml each of I and II; (C) Subject whole blood on day 15 of multiple dosing with one drop to each eye of a 2% ophthalmic solution of I; the content of I in 0.1 ml whole blood was 262.4 ng, concentration of II measured separately using 1 ml of whole blood was 6 ng/ml.

column switching valve was employed to eliminate this late eluting peak. The validation data for the column switching method are presented below.

3.2. Whole blood assay using column switching technique

Since the majority of the drug and metabolite in clinical samples were present in whole blood at relatively high concentrations, a less sensitive but more efficient and faster method for determination of I and II in whole blood was desirable. Using the procedure described in the Experimental section, a CS method with shorter run time (20 min *vs.* 28 min in the original assay) and higher LOQ (50 *vs.* 5 ng/ml), was developed and fully validated in the concentration range of 50–5000 ng/ml. The intra- and inter-day precision and accuracy data are presented in Tables 3 and 4, and representative chromatograms are shown in Fig. 3.

Typical equations for the standard line were $y = 8.099x - 11.080$ and $y = 2.858x + 8.909$ for I and II, respectively, using peak heights for calculations.

3.3. Analyses of samples from clinical studies

More than 5000 whole blood, plasma, and urine samples from various clinical studies with I were analyzed using the NCS and CS assays described above. As an example, representative concentrations of I and II in whole blood after topical ocular administration of I in selected human subjects participating in a pilot multiple dose clinical pharmacokinetic study obtained using NCS assay are presented in Table 5.

Table 3
Intra-day precision and accuracy of the assay of I and II in human whole blood using column switching technique

Spiked concentration (ng/ml)	I		II	
	C.V.[a] (%)	Accuracy[b]	C.V.[a] (%)	Accuracy[b]
50	2.1	103	3.4	105
100	2.9	99	3.3	99
200	0.7	99	0.7	99
500	2.3	97	1.1	97
1000	2.9	99	1.8	98
2000	2.1	100	2.5	100
4000	2.6	98	1.2	99
5000	0.7	99	0.7	99

[a]Coefficient of variation ($n = 5$).
[b]Expressed as (mean observed concentration)/(spiked concentration) × 100 ($n = 5$).

Table 4
Inter-day variability of the determination of quality control samples spiked with I and II using column switching assay

Spiked concentration (ng/ml)	I		II	
	Assayed concentration (ng/ml)	C.V.[a] %	Assayed concentration (ng/ml)	C.V.[a] %
200	185.2[b]	6.7	188.7[b]	7.0
	192.6[c]	3.9	184.2[c]	4.4
4000	3737.9[b]	3.7	3805.6[b]	3.0
	3833.2[c]	6.8	3850.0[c]	7.4

[a]Coefficient of variation ($n = 5$).
[b]Mean initial within-day analysis ($n = 5$).
[c]Mean inter-day analysis ($n = 6$) over a period of seven days.

Table 5
Concentration of I and II in whole blood of selected human subjects after topical ocular administration of 3% ophthalmic solution of I[a] using NCS assay

Subject No. Subject No.		Concentration (ng/ml)				
	Analyte	Day 1	Day 7	Day 14	Day 18[b]	Day 28[b]
101	1	91.0	1770.8	2724.6	2406.4	1963.6
	2	0.0	11.5	58.6	103.0	122.8
104	1	49.3	1165.1	1890.3	1726.3	1571.6
	2	0.0	8.3	27.1	36.7	46.4
105	1	47.6	870.9	1681.1	1598.7	1300.7
	2	0.0	0.0	19.7	27.8	41.1
109	1	19.0	854.4	1758.4	1809.5	1501.1
	2	0.0	6.2	20.9	29.1	38.4

[a]One drop of the ophthalmic solution to each eye, three times a day for a period of 14 days.
[b]Post-study samples, dosing ended on day 14.

4. Discussion

A major factor affecting the pharmacokinetics of all CAIs, including I, is binding to carbonic anhydrase in red blood cells (RBCs). Therefore, the measurement of the concentration of I in whole blood was primarily required to assess the pharmacokinetic behavior of I. Since the de-ethylated metabolite II was also found to bind strongly to RBCs present in whole blood, an assay for II in whole blood in the presence of I was also needed. In the latter stages of the pharmacokinetic program, similar assays in plasma and urine were necessary to assess the clearance of I and II from RBCs. Due to the relatively high molar absorption coefficient ($\epsilon \sim 13\ 000\ M^{-1}\ cm^{-1}$ at $\lambda = 252$ nm) of I in the accessible ultraviolet (UV) absorption region, all the methods described in this paper were based on HPLC with UV detection, and allowed reli-

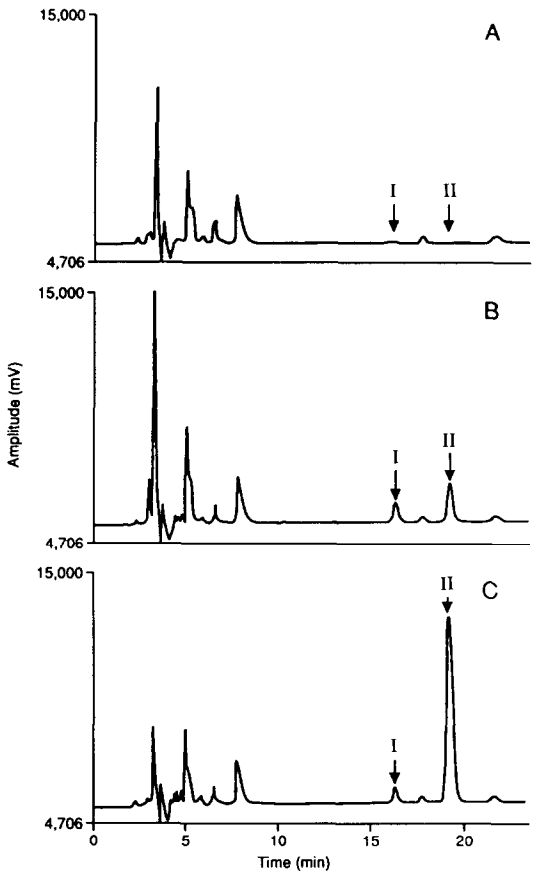

Fig. 3. Representative chromatograms of human whole blood containing I and II using CS assay. (A) Blank control blood; (B) Whole blood spiked with 200 ng/ml each of I and II; (C) Subject whole blood (diluted 1:2 with control whole blood before analysis) after approximately 12 months of dosing with one drop to each eye of a 2% ophthalmic solution of I; concentration of I and II was 1866.2 and 285.2 ng/ml, respectively.

able quantitation of I and II in biological fluids at low ng/ml levels.

Both I and II were isolated from the basified (pH 8) biological fluids using liquid–liquid extraction with a mixture of ethyl acetate–toluene–2-propanol, back extraction into a small volume of diluted phosphoric acid, and direct injection of an aliquot into the HPLC system. Several extracting solvents including methylene chloride, ethyl acetate, hexane, and toluene, and several of their combinations were utilized in the exploratory stages of assay development. All solvents were inadequate due to either poor extraction efficiency of I and/or II, or presence of interfering endogenous impurities eluting in the region where the compounds of interest eluted. The solvent system finally chosen for the assay gave chromatograms free of impurities, and high recoveries of I from whole blood, plasma and urine, and of II from urine. In the case of metabolite II, recoveries from whole blood and plasma were generally much lower than for I. Solid-phase extraction on C_8, C_{18} and cyano cartridges was additionally evaluated, but did not lead to improvement of the metabolite recovery. Since the recovery was constant in the whole concentration range studied, reliable quantitation of II could be performed at the same concentrations (LOQ = 5 ng/ml) as for I.

In the case of extraction from whole blood and plasma, an additional pre-treatment step with trichloroacetic acid, to lyse cell walls and denature the proteins, was required. Without this step recovery from whole blood and plasma was highly variable and unacceptably low.

The separation of I and II from each other and from endogenous impurities was achieved by ion-pair chromatography using octane- (NCS assay) or nonane-sulfonic acids (CS assay) under acidic conditions. A variety of mobile phase compositions were evaluated, and the selection of the final mobile phase and the concentration of the ion-pairing reagent was based on baseline separation of the parent drug and the metabolite from each other and from peaks of endogeneous impurities. Utilization of ion-pair chromatography was required to retain I and II on the column long enough to achieve the required separation. The difference in retention times of parent compound and the metabolite was *ca.* 3 min (Figs. 2 and 3) and was necessary to affect the separation of endogeneous interferences from the compounds of interest. However, a late eluting peak was always present in these ion-pairing systems, interfering with the integration of peaks of interest in consecutive injections. This late eluting peak was eliminated by increasing the run time to 28 min and placing this peak in the early region (solvent front) of the next

chromatogram (NCS assay). More than 4000 injections of biological fluid extracts were made on a single column, and deterioration in column performance was not observed. The late eluting peak in the CS assay was eliminated using the column switching system [5] consisting of two HPLC pumps, a 50-mm column (column 1), a 250-mm column (column 2), and an electrically actuated switching valve. The 50-mm column was used to separate both I and II from late eluting peaks, while the 250-mm column was used to separate the analytes from each other and from co-extracted endogenous impurities. The system was programmed so that both HPLC columns were in-line when the sample was injected. After I and II eluted from the 50-mm column, the columns were taken out of line. The analytes were separated from each other and from interfering substances on the 250-mm column, while the late eluting peaks from the 50-mm column were directed to waste.

The assays were utilized routinely for a period of almost three years in support of a variety of human pharmacokinetic studies. The methods were rugged, easy to transfer between analysts, and proved to meet the very stringent validation criteria required for analyses of samples from clinical studies.

5. Acknowledgements

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