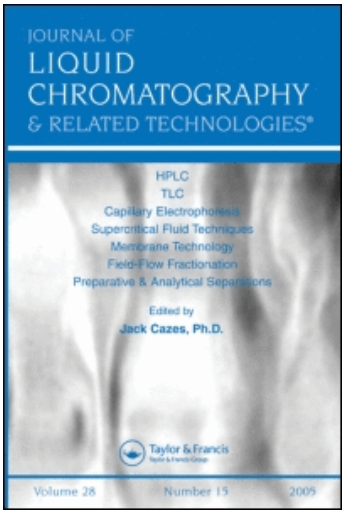

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Dovletoglou, Angelos , Thomas, Scott M. , Berwick, Lorrie , Ellison, Dean K. and Tway, Patricia C.(1995) 'Development of Practical HPLC Methods for Analysis and Quality Assessment of the Novel Carbonic Anhydrase Inhibitor MK-0507 and the Acetamidosulfonamide Intermediate', Journal of Liquid Chromatography & Related Technologies, 18: 12, 2337 — 2352
To link to this Article: DOI: 10.1080/10826079508013967
URL: http://dx.doi.org/10.1080/10826079508013967

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(12), 2337-2352 (1995)

DEVELOPMENT OF PRACTICAL HPLC METHODS FOR ANALYSIS AND QUALITY ASSESSMENT OF THE NOVEL CARBONIC ANHYDRASE INHIBITOR MK-0507 AND THE ACETAMIDOSULFONAMIDE INTERMEDIATE

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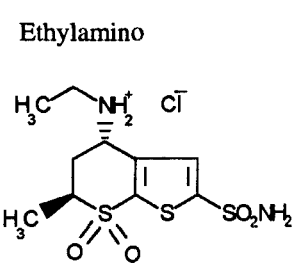
ABSTRACT

MK-0507 (Dorzolamide HCl, (4S,6S)-4-Ethylamino-5,6-dihydro-6-methyl-4*H*-thieno-[2,3-*b*] thiopyran-2-sulfonamide 7,7-dioxide hydrochloride) is the first topically active, water-soluble, carbonic anhydrase inhibitor to be developed for the treatment of glaucoma and ocular hypertension. Dorzolamide HCl is an effective and well tolerated agent as monotherapy for patients who cannot tolerate ophthalmic beta-blockers, and for those who need add-on therapy to beta-blockers.[1] The steps taken in the development and validation of a fast and rugged reverse-phase HPLC method for the analysis and quality assessment of MK-0507 drug substance and acetamidosulfonamide intermediate are described. Four columns were used during the development: Du Pont Zorbax C-18, Rainin Microsorb C-8, Perkin Elmer CR-C8 and YMC4. The last two columns showed excellent resolution, peak shape, and precision. The selected HPLC method for acetamidosulfonamide, using the Perkin Elmer CR-C8 column, was validated with respect to linearity, limit of detection (LOD) and limit of quantitation (LOQ). The selected HPLC method for the MK-0507 drug substance using the Perkin Elmer CR-C8 column was validated with respect to linearity, LOD, LOQ, precision on an injection-to-injection basis, and on a day-to-day basis, selectivity and accuracy.

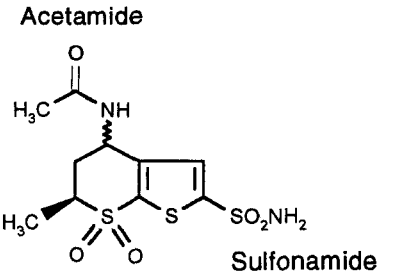
The method is rugged with respect to solution stability, variations in buffer concentration, flow rate, column-to-column variability and column temperature. The relative response factor for the *cis*-isomer of MK-0507 and the desethyl impurity were also determined in order to quantitatively measure the levels of these impurities in MK-0507. There are three possible stereoisomers of MK-0507 (4S,6S): the enantiomer 4R,6R and the *cis*-diastereoisomers 4S,6R and 4R,6S. The chiral HPLC method separates the two *trans*-diastereoisomers, and the two *cis*-diastereo-isomers; however, in the same chiral method the 4R,6R and 4R,6S stereoisomers coelute.

INTRODUCTION

A number of sulfonamides have been separated by using reverse- or normal-phase HPLC methods.[2] To our knowledge, there is no documentation of any separation of sulfonamide that contains a sulfone and/or an acetamide moiety. MK-0507 (I) is a sulfonamide that contains a sulfone and ethylamino group. Acetamidosulfonamide (II) contains a sulfone and an acetamide group.



(I)



(II)

The goals of analytical development of the HPLC method for the MK-0507 drug substance and acetamidosulfonamide were to provide methodology for characterization of purity and impurities during process development, stability studies, pilot plant campaigns and manufacturing. Also, the benefits of applying a single chromatographic method to monitoring completion of reactions were evaluated.

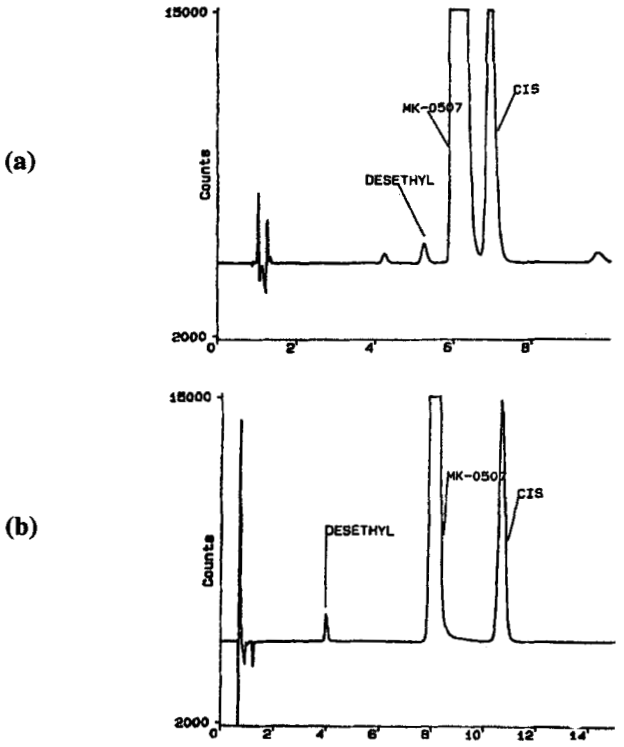


FIGURE 1. HPLC chromatograms of a mixture of 90:10:1 (w/w/w) MK-0507: *cis*-isomer of MK-0507:Desethyl Using (**a**) Microsorb C-8 and (**b**) Perkin Elmer CR-C8 Column.

The initial HPLC impurity profile involved a reverse-phase column (Du Pont Zorbax C-18) with gradient elution (aqueous acetate buffer (pH=5.0)/CH_3CN mobile phase). A second HPLC method was implemented using a Microsorb C-8 column (aqueous acetate buffer (pH=4.5) /CH_3CN mobile phase) (Figure 1a). The latter system showed improved peak shape and demonstrated better resolution of MK-0507 and the *cis*-isomer (R_s = 2.2 - 2.4). Both of these reverse-phase methods resulted in broad peaks (T_f >1.5), due to presence of two

amine groups (sulfonamide and ethylamino group). In a typical reverse-phase system, it is known that the interactions of amine groups with the surface Si-OH groups (silanols) cause tailing.[3] A new HPLC assay was developed by switching to a better end-capped column (Perkin Elmer CR-C8) and by adding triethylamine (TEA) to the mobile phase. These changes resulted in better peak shape (T_f=1.0-1.2) and greatly improved resolution (R_s = 5.6 - 6.4) between the two isomers (Figure 1b). The chromatographic performance of a second column (YMC4) was fully evaluated and compared to the Perkin Elmer CR-C8 column, and equivalent results were obtained (R_s = 6.0 - 7.0, T_f = 1.0-1.5).

MK-0507 contains two chiral centers with four possible stereoisomers: MK-0507 (4S,6S), the enantiomer 4R,6R and the diastereoisomers 4S,6R and 4R,6S.[4] The normal-phase chiral HPLC method was originally designed to control the level of the 4R,6R enantiomer.[5] Also, to date, no experimental evidence exists to demonstrate that both *cis*-isomers (4R,6S and 4S,6R) are controlled by the reverse-phase HPLC test. In this work it is determined that the stereochemical configuration is controlled by a combination of the normal-phase chiral HPLC and reverse-phase HPLC methods.

EXPERIMENTAL SECTION

Materials

MK-0507 was prepared in the *Departments of Medicinal Chemistry and Process Research, Merck Research Laboratories.*[6] The 4R,6R diastereoisomer of MK-0507, was provided by Mr. P. Sohar (*Process Research, Merck Research Laboratories*). The two enantiomers of *cis*-isomer of MK-0507, *cis*(+), 4S,6R: L-685,973 and *cis*(-), 4R,6S: L-685,974, were synthesized by Dr. Ponticello and his group (*Medicinal Chemistry, Merck Research Laboratories*). The chiral derivatizing reagent, (*S*)-(-)-α-methylbenzyl isocyanate, was purchased from Aldrich. All the solvents (Fischer Scientific) used were HPLC grade.

Solutions, Sample Preparation and Chromatographic Conditions

A 60 mg sample of MK-0507 (CAS Number: 130693-82-2) was dissolved into 100 mL of 20:80 (v/v) MeOH:H_2O. Using the Perkin Elmer CR-C8 and YMC4 columns, the mobile phase composition is: A: 1.0 mL of HPLC grade $(CH_3CH_2)_3N$ (TEA) and 1.0 mL of HPLC grade acetic acid (HOAc) into 1 L of HPLC grade H_2O, and B: HPLC grade CH_3CN. This method is very sensitive to changes in pH. The pH of solution A should be between 4.4 and 4.6. MK-0507 and acetamidosulfonamide were analyzed using the PE CR-C8 column with a mobile phase of 100% A for 10 min and then changing to 50% A in 20 minutes, and with UV detection at 254 nm. The injection volume was 10 µL, with a flow rate of 1.5 mL/min at ambient temperature. The data points collected for three samples indicate that MK-0507 is stable in the recommended diluent for at least seven days at room temperature. The Du Pont Zorbax C-18 is used with 98:2 (v/v) 0.02 M aqueous acetate buffer ($AcONH_4$) (pH=5.0):CH_3CN mobile phase at 2.0 mL/min. The Microsorb C-8 column is used with 98:2 (v/v) 0.02 M aqueous acetate buffer ($AcONH_4$) (pH=4.5):CH_3CN mobile phase at 1.8 mL/min.

Prior to an injection on a silica column, the two enantiomers of the *cis*-isomer of MK-0507 were derivatized with (*S*)-(-)-α-methylbenzyl isocyanate to form the urea derivatives 4S,6R(S) and 4R,6S(S). After the chiral derivatization, the mixture of 4R,6S(S) and 4S,6R(S) were separated under non-chiral chromatographic conditions (normal-phase Zorbax SIL column) with a *tert*-butyl methyl ether/acetonitrile/heptane mobile phase. The chromatographic conditions for the chiral assay of the *cis*-isomer of MK-0507 are: Zorbax SIL column (250 x 4.6 mm) and mobile phase of 65:35 (v/v) methyl tert-butyl ether (containing 3.0% CH_3CN and 0.3% H_2O):heptane. The flow rate is 2 mL/min, λ = 254 nm, injection volume is 10 µL, sample concentration is 2.0 mg/mL at ambient temperature, and retention times are 4S,6R(S) ~12 min, and 4R,6S(S) ~16 min.

Chromatographic/system suitability parameters, such as tailing factor (T_f) and resolution (R_s), etc., were calculated using Nelson Access*Chrom GC/LC Data System suitability software <USP method>. Digital spectra, from 200 nm to 440 nm, were taken using a diode array UV detector. The spectra for four points across the main peak were baseline corrected and the first and second derivatives were taken. The baseline-corrected spectra were normalized and the derivative plots were overlayed.

Instrumentation and Measurements

The HPLC system consisted of a SP8700 Spectra-Physics gradient pump, a Spectroflow 757 (Kratos Analytical) UV-Vis detector or a Applied Biosystems 759A UV-Vis detector and a SP8775 Spectra-Physics autosampler using a Perkin-Elmer CR C-8, particle diam 3 μm, pore diam 60 Å, length 8 cm, ID 3.0 mm column; YMC4, particle diam 3 μm, length 15 cm, ID 4.6 mm column; Du Pont Zorbax C-18, particle diam 5 μm, pore diam 120 Å, length 8 cm, ID 4.6 mm column; and Rainin Microsorb C-8, particle diam 5 μm, length 15 cm, ID 4.6 mm columns.

Chemical and Physical Stress Conditions

Acetamidosulfonamide: A 500 mg sample was stressed at 40 °C/75% relative humidity for 1 mo, and three 100 mg samples were thermally stressed at 40, 60 and 100 °C for 24 h. Four solutions of the stressed materials were prepared (1 mg/mL) and analyzed using diode array detection. The spectra for four points across the main peak were baseline corrected and the first and second derivatives were taken. The baseline-corrected spectra were normalized and the derivative plots were overlayed.

MK-0507: *Acid Stress:* MK-0507 was stored in 2*N* HCl solution at ambient temperature for 48 h. *Base Stress:* A sample of MK-0507 was stored in 2% (w/w) NaOH solution at ambient temperature for 48 h. *Peroxide Stress:* MK-

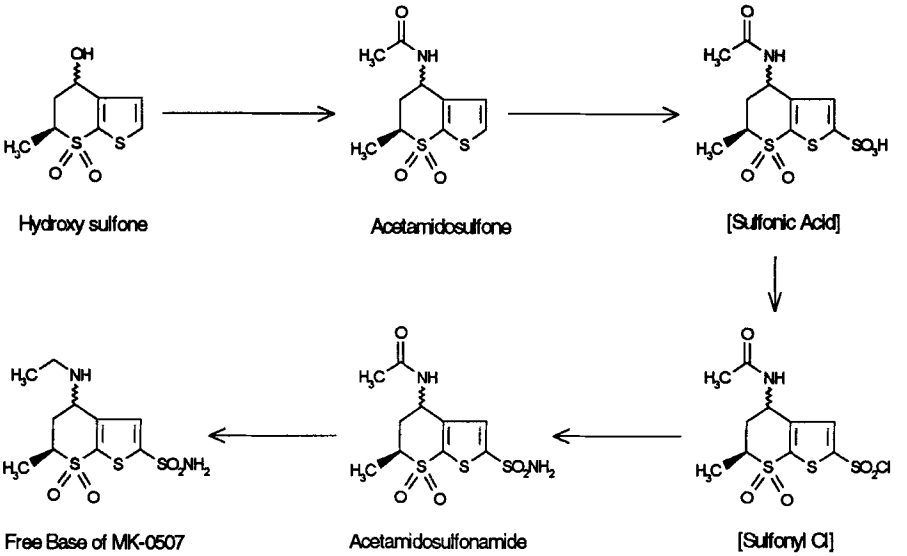
0507 was stored in a concentrated peroxide solution at ambient temperature for 2 h. *Thermal Stress:* A sample of MK-0507 was thermally stressed at 150 °C for 12 h. *UV Light Stress and White Light Stress:* Using a Rayonet Photochemical Reactor model RPR-200, equipped with nine ultraviolet bulbs (24 W, 90% in the 3500 angstrom range) or equipped with eight fluorescent bulbs (8 W, cool white light, Sylvania Fluorescent cat# F85T/CW), MK-0507 was exposed for 24 h. *Final Crystallization Solvent Stress:* A 1% aqueous solution of MK-0507 was prepared and stored at 80 °C for 18 h.

RESULTS AND DISCUSSION

In-process HPLC methods were developed for the synthesis of MK-0507.[7] All the in-process tests were designed to determine the impurity profile and the completion of reaction. The starting material for the synthesis is hydroxy sulfone ((6S)-5,6-Dihydro-4-hydroxy-6-methyl-4H-thieno[2,3-b]thiopyran-4-ol-7,7-dioxide, HS). The complete synthesis is shown in Scheme I.

Acetamidosulfonamide Validation

The assay for acetamidosulfonamide was validated and the detector response was found to be linear over the concentration range of 0.0001 to 0.96 mg/mL. We estimate the detection limit to be 0.0001 mg/mL or 1.45 ng (0.02% relative to the recommended sample concentration 0.5 mg/mL sample concentration). This estimate is based on data collected from the linearity study and a typical chromatogram with 1.45 ng injected on-column. The signal-to-noise level is 4:1 at this concentration. We estimate the limit of quantitation to be 0.0002 mg/mL (0.06% relative to the 0.5 mg/mL sample concentration). This estimate is based on data collected from the linearity study. Injection precision was demonstrated by dissolving a sample of acetamidosulfonamide in the recommended diluent and injected 11 consecutive times, resulting in a relative standard deviation of 0.2%.



Scheme I

Day-to-day precision was demonstrated by preparing three weighings of the reference standard and two weighings of acetamidosulfonamide on each of three days, resulting in a relative standard deviation of <0.5%. Acetamidosulfonamide is stable over a four day period in the proposed diluent. The method is selective, as judged by photo diode array detection and as demonstrated by the resolution of the *cis*- and *trans*-acetamidosulfonamide.

The method is rugged to variations in mobile phase composition and flow rate. The influence of mobile phase variations in the separation of *cis*- and *trans*-acetamidosulfonamide was examined. By varying the mobile phase composition, it was found that the optimum ratio is 6% CH_3CN and 94% buffer solution. Excellent k' and T_f values were obtained at flow rates of 1.0, 1.5 and 2.0 mL/min. The optimum resolution and number of theoretical plates was observed at 1.5 mL/min.

Using the PE CR-C8 and YMC4 column, the typical retention times of the in-process intermediates shown in Figure 2 are: 3-positional isomer of acetamidosulfonamide (3-AASA, ~6 min), *cis*-acetamidosulfonamide (*cis*-AASA), *trans*-acetamidosulfonamide (*trans*-AASA) and bis-acetamidosulfonimide (dimer). The *cis*-acetamidosulfonamide has a slightly higher response factor (1.07) than *trans*-acetamidosulfonamide.

Stress Studies of Acetamidosulfonamide

During the stress studies of acetamidosulfonamide diode array detection found no indication of impurities with a different UV chromophore coeluting with the main peak. A sample of acetamidosulfonamide was stressed at 40 °C/75% relative humidity for 1 mo, and three samples of acetamidosulfonamide were stressed at 40, 60 and 100 °C for 24 h. No new impurities were formed and levels of existing impurities remained the same. The selectivity of the method was demonstrated by using diode array detection to analyze (thermally stressed) samples of acetamidosulfonamide. No change in the physical appearance of the samples was observed and diode array detection revealed no evidence of the formation of thermal degradates.

MK-0507 Drug Substance Validation

Using the PE CR-C8 column, the detector response for the MK-0507 peak is linear over the concentration range of 0.0001 to 1.46 mg/mL. The correlation coefficient over this range is 0.999997. The sample concentration of 0.5 mg/mL is well within the linear range of this method and there is no bias toward low level impurities. The detection limit is 1.45 ng (0.02% relative to the recommended 0.5 mg/mL sample concentration). The signal-to-noise level at 1.45 ng on-column injection level is 4:1. Based on the data collected in the linearity study, the limit of quantitation is 0.0003 mg/mL (0.06% relative to the 0.5 mg/mL sample concentration). Three consecutive injections of the 0.0003 mg/mL solution averaged 3028 area counts with an RSD of 5.8%. The method

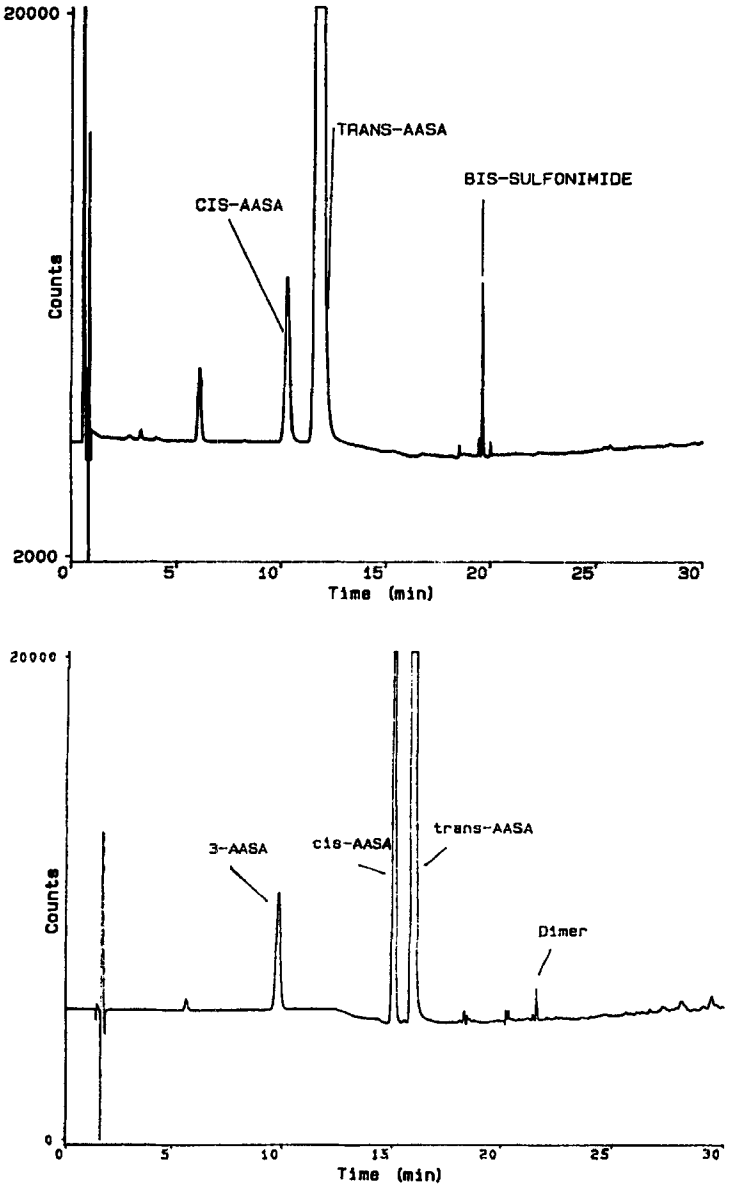


FIGURE 2. HPLC chromatograms of Acetamidosulfonamide Using (**a**) Perkin Elmer CR-C8 and (**b**) YMC4 Column.

has acceptable sensitivity to low-level impurities and has a limit of quantitation that meets our requirement of 0.10% minimum. The HPLC assay of a typical lot of the drug substance prepared in the pilot plant (99.8%) is accurate compared to the silver nitrate titration (99.9%), perchloric acid titration (99.9%), and phase solubility analysis (100%, Slope = 0.0 +/- 0.1).

The method is precise on an injection-to-injection and on a day-to-day basis. MK-0507 was injected 11 consecutive times, resulting in a relative standard deviation of 0.1% for the MK-0507 peak. Three samples were prepared on three different days, and each solution was injected in triplicate each day. The overall precision is acceptable, with an RSD of < 0.5% for the MK-0507 peak.

The effect of buffer concentration, pH, flow rate, temperature, and column-to-column variability on the method was studied. The mobile phase was studied as a function of the triethylamine (TEA) and acetic acid (AcOH) concentrations. The concentration of TEA and AcOH varied by 0.05% from 0.05% to 0.20% (v/v) each. No significant change in any of the chromatographic parameters was observed (Table I).

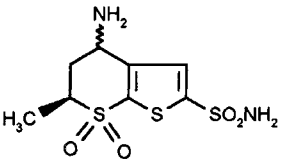
The pH of the mobile phase varied over the range of 3.8 to 5.0 (Table I). We found that the pH significantly influences the capacity factor (k'), number of theoretical plates (N) and peak tailing factor (T_f), (Table I). Changes were observed in the resolution between MK-0507 and *cis*-isomer of MK-0507, and also between the MK-0507 and the desethyl impurity (a known impurity formed during the MK-0507 process). In all cases, the resolution was acceptable (Table I).

The resolution of MK-0507 was studied as function of the flow rate over the range of 1.0 to 2.0 mL/min. High values of k' were obtained at 1.0 mL/min (MK-0507, k'=17.7) and high back pressure (>3000 psi), and increased peak tailing was observed at 2.0 mL/min (MK-0507, T_f = 2.0). The optimum flow rate was 1.5 mL/min.

TABLE I: Chromatographic Parameters of Desethyl (des), MK-0507 and *cis*-isomer of MK-0507 (*cis*) as a Function of Buffer Concentration and pH Using the PE CR-C8 Column (Flow Rate = 1.5 mL/min).

Conc.[1] Vol%	(pH)	k' des	k' 0507	k' *cis*	T_f 0507	N 0507	R_s[2]	R_s[3]
0.05		4.8	11.0	14.8	1.0	7344	>10	6.1
0.10	(4.5)	4.8	10.3	14.1	1.0	7406	>10	6.2
0.15		4.6	9.8	13.4	0.9	7400	>10	6.3
0.20		4.9	10.0	13.7	0.9	7527	>10	6.4
	(3.8)	3.5	5.1	7.8	1.2	5829	5.9	7.1
	(5.0)	7.4	24.2	30.4	0.9	8266	>10	4.8

1- TEA and AcOH each
2- Resolution between desethyl (des) and MK-0507
3- Resolution between MK-0507 and *cis*-isomer of MK-0507 (*cis*)



desethyl

The resolution of MK-0507 was studied as function of column temperature over the range of 25 to 40 °C. A decrease in k' (10.8 to 8.3) and R_s (7.1 to 5.2) for MK-0507 was observed as the column temperature increased, but the k' and R_s remained within the recommended operating parameters. The number of theoretical plates remained constant (~10,000 +/- 200).

The relative response factor for desethyl relative to MK-0507 is 1.20 and for *cis*-isomer of MK-0507 relative to MK-0507 is 0.98. The level of these impurities can be reliably measured using this assay.

The pH of the mobile phase has a significant influence in the retention of MK-0507 and *cis*-isomer of MK-0507. As the pH increases, the *N* decreases. The retention of the desethyl impurity is influenced, but to a lesser degree. The peak shape and the number of theoretical plates are also pH dependent.

Stress Studies of MK-0507 Drug Substance

Selectivity of the method was demonstrated by resolving impurities formed under the following stress conditions: 1) acid stress, 2) base stress, 3) peroxide stress, 4) severe thermal stress, 5) UV light stress, 6) white light stress and 7) stress of final crystallization solvent. In all of the stress studies listed below, no impurities were detected, by diode array detection, to have different UV chromophore coeluting with the main peak. Under acid stress and peroxide stress, the impurity formed at relative retention time (RRT) 0.06 (in solvent front) has a significantly different chromophore than that of MK-0507. Under base stress the impurity formed at RRT 1.35 is the *cis*-isomer of MK-0507. During thermal stress, a change in the physical appearance of the sample was observed: the color of the stressed sample was light tan as opposed to the white non-stressed material. A 1 mg/mL solution of the stressed material was prepared and analyzed using the diode array system and no decomposition was observed. No change in the physical appearance of the sample was observed under UV light stress, white light stress and final crystallization solvent (H_2O) stress. A 1 mg/mL solution of the stressed material was prepared and analyzed using the diode array system and no decomposition was observed.

MK-0507 Process Intermediates

A sample of MK-0507 was spiked with desethyl and with all the isolated process intermediates. All the compounds are well resolved from the main peak

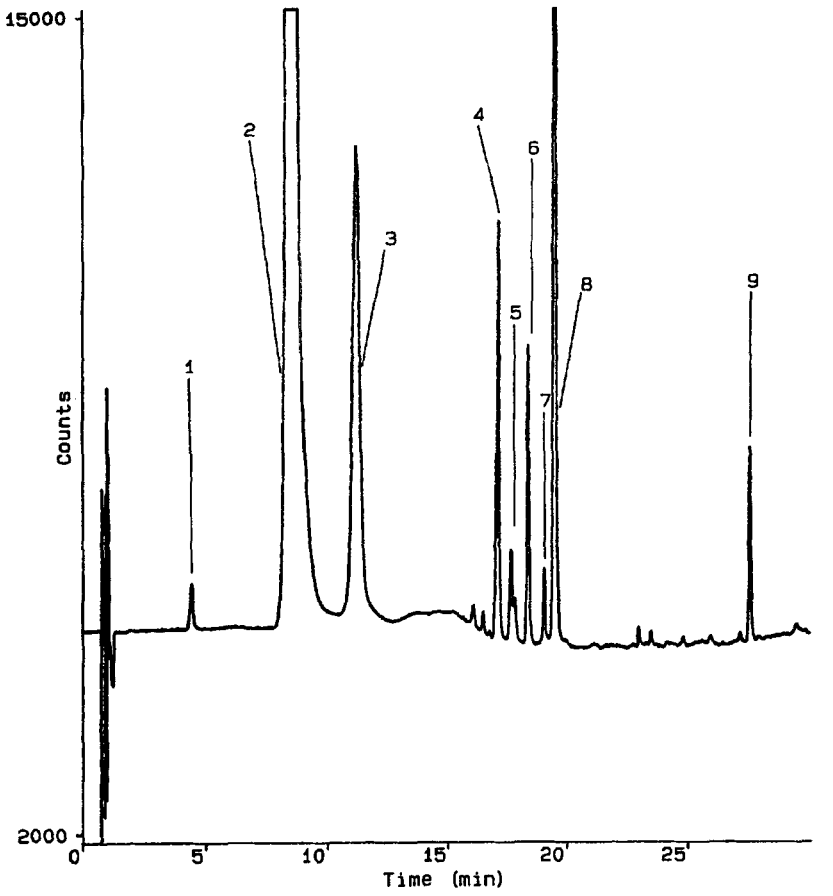


FIGURE 3. Resolution of Process Intermediates and Process Impurities Using PE CR-C8. 1 -Desethyl (RRT 0.52); 2 -MK-0507 (RRT 1.00); 3 -*cis*-isomer of MK-0507 (RRT 1.32); 4 -*cis* & *trans*-Sulfonic Acid (RRT 2.01) 5 -*cis*-Hydroxysulfone (RRT 2.08); 6 -*trans*-Hydroxysulfone (RRT 2.16); 7 -cis-Acetamidosulfone & *cis*-Acetamidosulfonamide (RRT 2.24); 8 -*trans*-Acetamidosulfone & *trans*-Acetamidosulfonamide (RRT 2.30); 9 -*trans*-Sulfonyl chloride (RRT 3.23)

(Figure 3). Each of the desethyl solutions was diluted 1000X to simulate a 0.1% level impurity in a typical MK-0507 sample preparation.

Stereoisomers

The chiral separation of MK-0507 (4S,6S) and its enantiomer (4R,6R) has been validated (see experimental section). This method also separates the two derivatized enantiomers (4R,6S(S), 4S,6R(S)) of *cis*-isomer of MK-0507. The order of elution and peak assignmentswere established by injecting authentic samples of the four derivatized stereoisomers. The chromatographic behavior of the two *cis*-enantiomers is in good agreement with the system suitability test currently used in the manufacturing of MK-0507 to demonstrate resolution between MK-0507 and *cis*-isomer of MK-0507. Early in the development of the chiral test, evidence suggested that either the 4R,6S diastereoisomer or the 4S,6R diastereoisomer co-elutes with the 4R,6R enantiomer. An attempt to separate the mixture of all four stereoisomers (4S,6S, 4S,6R, 4R,6S and 4R,6R) under the same conditions resulted in chromatography with three peaks. The first peak is MK-0507, the second is 4S,6R and the third is both the 4R,6S and 4R,6R stereoisomers. The *cis*-isomer level can be determined directly from the reverse-phase HPLC method and the percent 4S,6R level can be determined directly from the chiral HPLC method. The percent 4R,6S can be calculated by subtracting the percent 4S,6R from the *cis*-isomer level.

CONCLUSION

A simple, accurate and reproducible HPLC method was developed for separating sulfonamides that contain a sulfone and/or an acetamide and/or ethylamino moiety. The limit of quantitation is <0.1%, and the limit of detection is 0.03% of the sample concentration. The method is capable of separating all nine starting materials, process intermediates and the final product. The same method can be used to successfully monitor all the steps for the synthesis of

MK-0507. A combination of methods is required to monitor the stereoisomer levels.

ACKNOWLEDGMENTS

Ms. J. Vandrilla for performing the phase solubility experiments.

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Received: January 20, 1995
Accepted: January 25, 1995