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Determination of ML-1035 Enantiomers in Plasma by Chiral High Performance Liquid Chromatography

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ABSTRACT

ML-1035, is a gastroprokinetic agent structrually related to metoclopramide. Because ML-1035 contains an asymmetric chiral sulfoxide moiety, a chiral HPLC method was developed to separate and quantitate its R and S enantiomers in plasma. The ML-1035 enantiomers present in plasma were extracted with dichloroethane under alkaline conditions. The extract was evaporated to dryness and reconstituted in the mobile phase. The samples were chromatographed on a Chiralcel OD HPLC column with hexane: absolute ethanol (1% TEA) with a ratio of 1:1 (v/v) as the mobile phase. The enantiomers of the unchanged drug were quantified by fluorescence detection (ex: 310 nm, em: 350 nm).

The method provided a linear response for both enantiomers over a concentration range of 25 (limit of quantitation)to 2500 ng/ml with correlation coefficients of 0.9987 or greater. The interassay precision was 9.5% or less and the accuracy ranged form 93.9% to 103.4% of the theoretical value. This method was utilized in determining the plasma concentrations of the R and S enantiomers following oral and intravenous administration of R or S enantiomers to dogs. The method was also adapted to measure enantiomer levels from in vitro reaction mixtures so that the possibility of metabolic inversion could be assessed. The data suggest that no significant level of inversion between the enantiomers occurred either in vivo or in vitro.

Key Words: Enantiomers, Chiral Seperation, Plasma, HPLC.

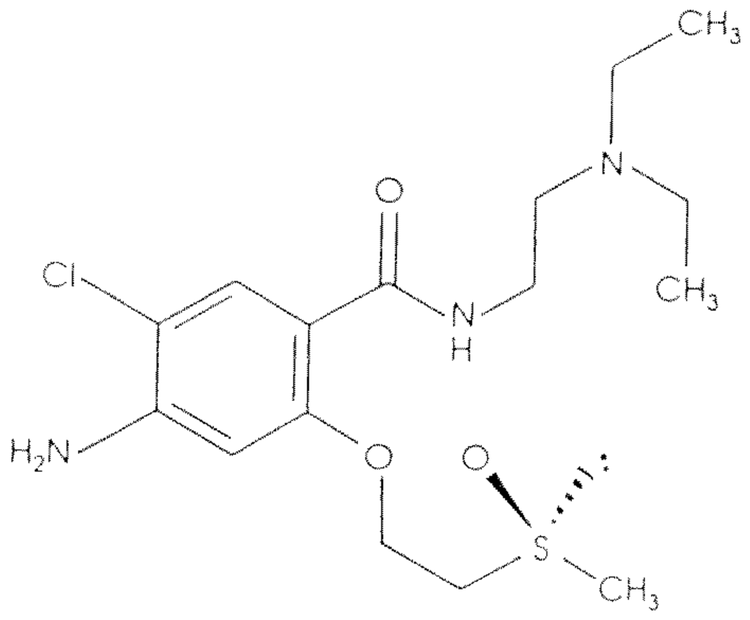
INTRODUCTION

ML-1035 is a gastroprokinetic agent which is structurally similar to metoclopramide, the widely used antiemetic and stimulant of upper gut motility [1-3]. ML-1035 is a racemic mixture with its chiral center in the sulfoxide moiety (Figure 1). Therefore, the objectives of this study were to develop a sensitive and selective chiral chromatographic method for quantitating the R and S enantiomers of ML-1035 in plasma, and then to determine the pharmacokinetic profile of the enantiomers following oral and intravenous administration to dogs. Further, the possible inversion of the R and S enantiomers was also investigated both in vitro and in vivo.

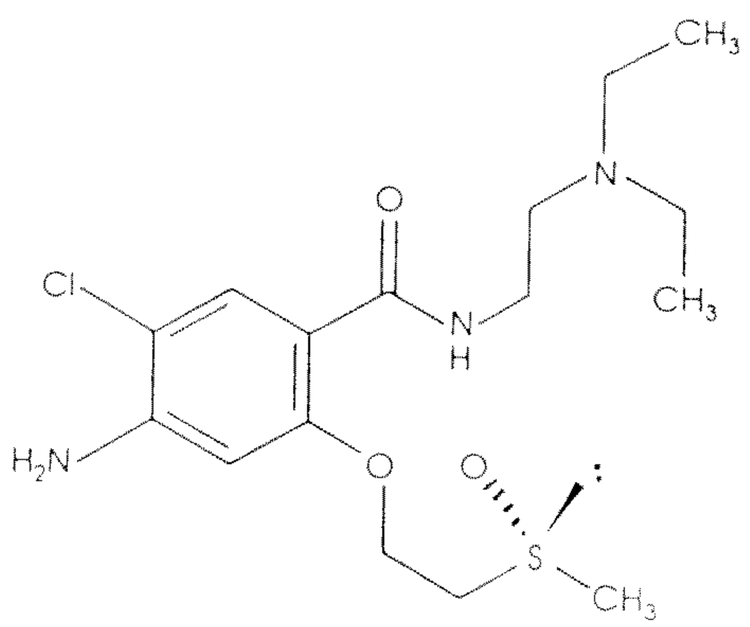
MATERIALS AND METHODS

1. *Materials and Reagents*

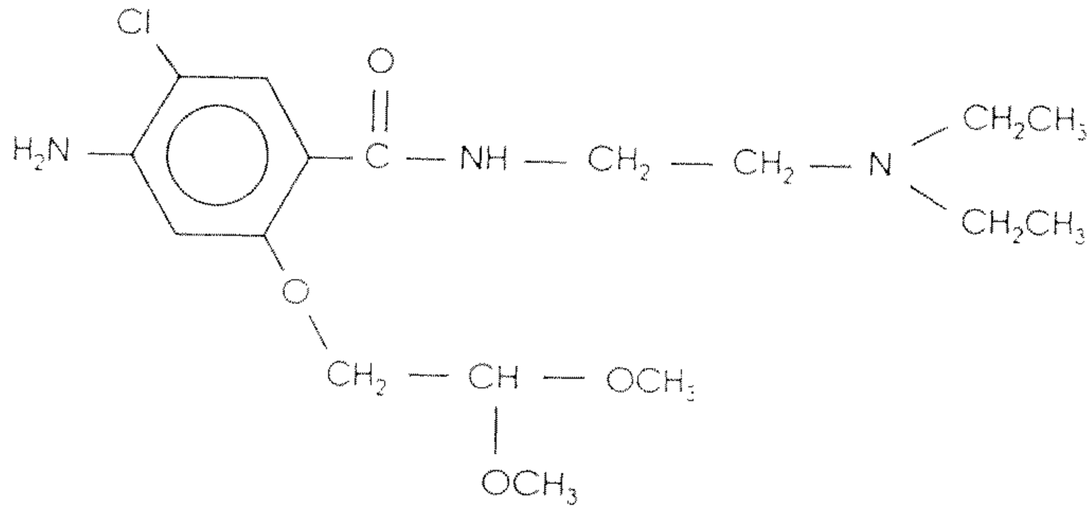
HPLC grade methanol, hexane, and dichloroethane were purchased from Buridck and Jackson (Muskegon, MI). Ethyl alcohol (absolute) was purchased from Quantum Chemicals Corporation (Tuscin,



ML-1035 (+)-S-4-amino-5-chloro-2-(methylsulfonyl) ethoxy)-N-(2-(diethylamino)ethyl)benzamide)



ML-1035 (−)-R-4-amino-5-chloro-2-(2-(methylsulfonyl) ethoxy)-N-(2-(diethylamino)ethyl)benzamide



Internal Standard

Figure 1. Absolute configuration of ML-1035 and the structure of internal standard BMY-25795.

IL). Triethylamine was purchased from Aldrich Chemical Company (Milwaukee, WI). BMY-25795 (Figure 1), obtained from the Bristol-Meyers Squibb Company, was used as the internal standard.

II. *Preparation of Standard Solutions*

Stock solutions of the R and S enantiomers and the internal standard BMY-25795 were prepared by weighing 10 mg of each into separtate 10 ml volumetric flasks and brought to volume with methanol. Serial dilutions of the stock were made in methanol to obtain working standard solutions of 0.01, 0.001, and 0.0001 mg/ml. Similarly, the internal standard working standards were prepared by diluting the stock to 0.01 mg/ml in methanol. All standards were stored at 0℃ to 5℃.

III. *Extraction of Plasma Samples and Calibration Standards*

The plasma calibration standards were prepared by spiking 1 ml of control dog plasma with the R and S enantiomers at concentration levels of 0, 25, 50, 100, 250, 500, 1000, 2500 ng in 15 ml screw-capped glass tubes. The internal standard, BMY-25795, was added at 500 ng by pipetting 50 μl of 0.01 mg/ml standard solution to plasma samples and calibration standards.

The extraction of the R and S enantiomers from plasma was initiated following the addition of 0.5 ml of 0.2 M Na_2CO_3 saturated with $(NH_4)_2HPO_4$ and 7 ml of dichloroethane. Tubes containing samples and calibration standards were slowly shaken for 30 minutes on a mechanical shaker and cetrifuged at 3000 rpm for 15 minutes. The top aqueous layer was aspirated to waste and the dichloroethane layer was evaporated to dryness in a Savant SpeedVac Concentrator (Farmingdale, NY). The dried samples and standards were reconstituted in 0.25 ml of hexane:ethanol (1:1 v/v) mobile

phase. The reconstituted samples were transferred to HPLC vials and a 200 μl aliquot was injected into an HPLC system.

IV. *Chromatographic Equipment and Conditions*

The chiral HPLC analysis was performed on a Perkin-Elmer modular liquid chromatographic system (Perkin-Elmer Corporation, Norwalk, CT) equipped with a LC-410 quateunary pump, ISS-100 autoinjector, and a LS-4 fluorescence detector. The detector was set to 310 nm excitation and 350 nm emission wavelengths. A Beckman PeakPro data system was used to collect, integrate, and analyze the chromatographic data.

The chromatographic separation of ML-1035 enantiomers was based on a Pirkle-type interaction [4,5]. The separation utilized a cellulose carbamate bonded silica gel column (5 μ Chiralcel OD, 4.6 mm x 250 mm from Diacel U.S.A. Inc. Fort Lee, NJ) along with a CN guard column (2 mm x 20 mm, 5 μ, from Alltech Associates, Deerfield, IL).

The mobile phase was prepared by mixing 10 ml of triethylamine and 990 ml of absolute ethanol. This ethanol solution was mixed online with n-hexane with a ratio of 1:1 (v/v) at a flow rate of 0.7 ml per minute. Both mobile phase solutions were degassed with helium prior to use.

V. *In Vivo and In Vitro Studies*

The utility of this method was determined by analyzing samples from *in vivo* and *in vitro* studies designed to measure the pharmacokinetic profile and possible metabolic inversion.

The R and S enantiomers of ML-1035 were administered to different dogs (n = 4) by a single I.V. dose of 2.5 mg/kg or an oral dose of 20 mg/kg. Blood samples were collected at 0, 0.083 (I.V. only), 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24 and 48 hours postdose, Plasma was separated by centrifugation and the samples were stored in a -20℃ freezer until analysis.

In vitro, the R and S enantiomers were incubated separately with liver 10,000 G supernatant (10S) from rat and dog. The incubate contained 0.1 mM of R or S enantiomer, rat or dog 10S equivalent to about 0.6 gm

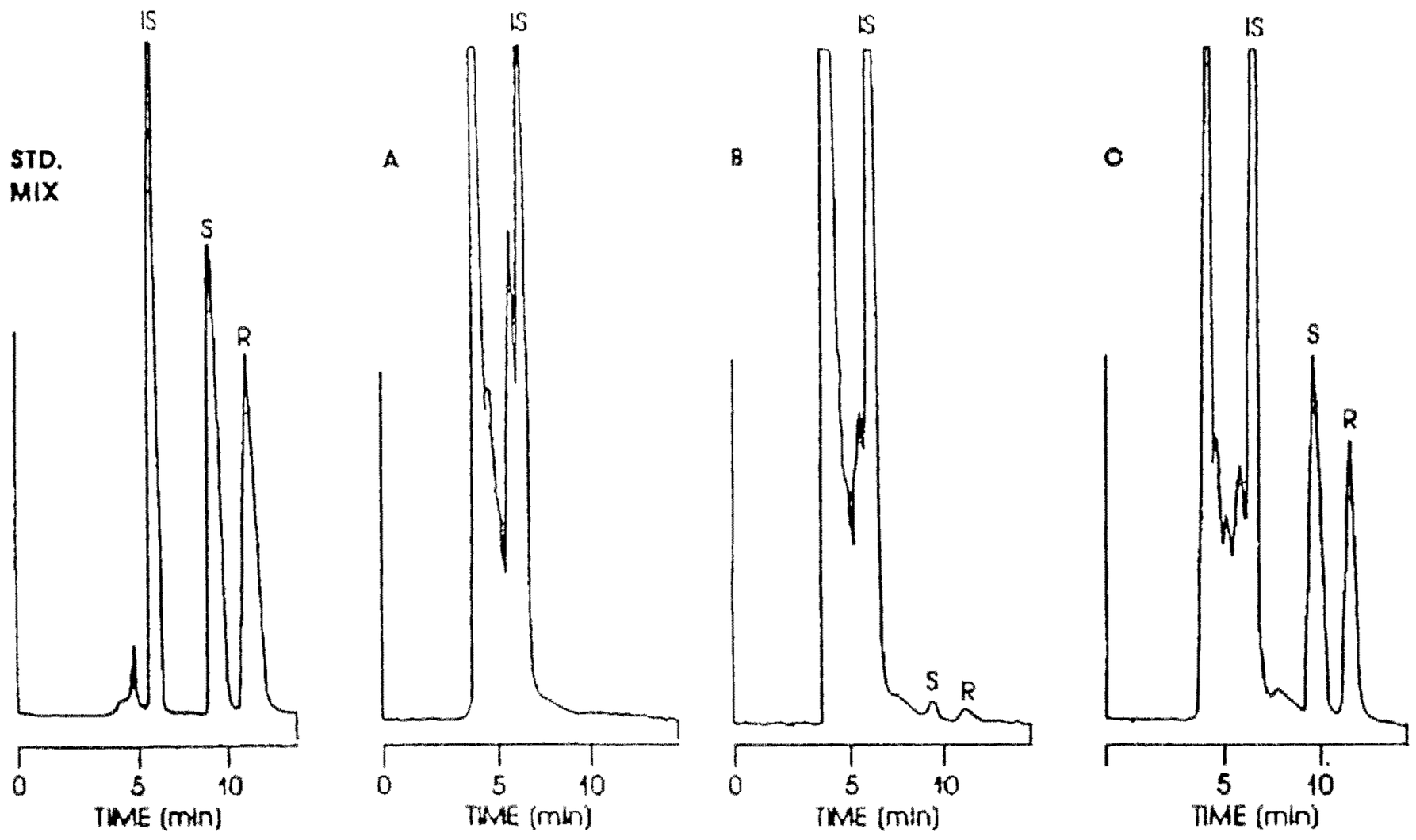


Figure 2. Chromatograms of spiked dog plasma at concentration levels of A) 0 ng/ml, B) 25 ng/ml, and C0 500 ng/ml of ML-1035 "R"and "S"enantiomers.

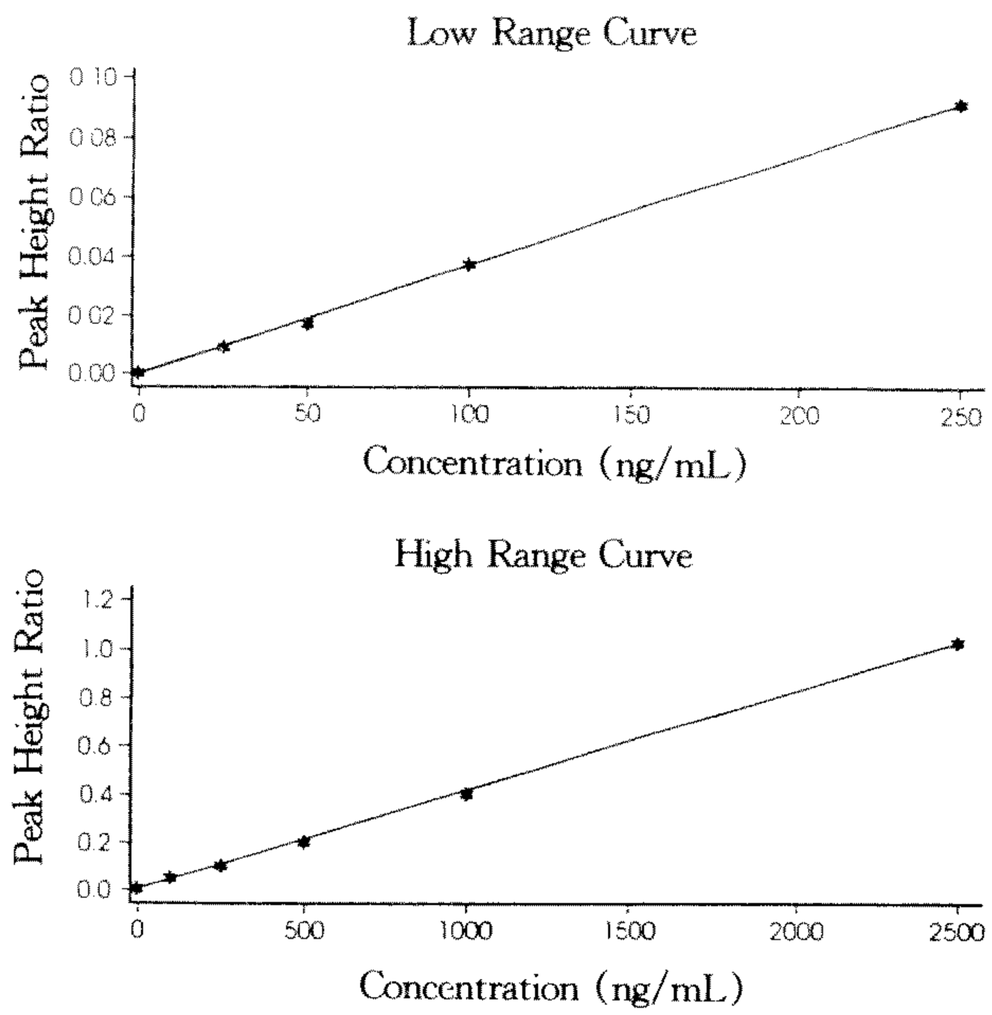


Figure 3. Calibration curves for R enantiomer in dog plasma.

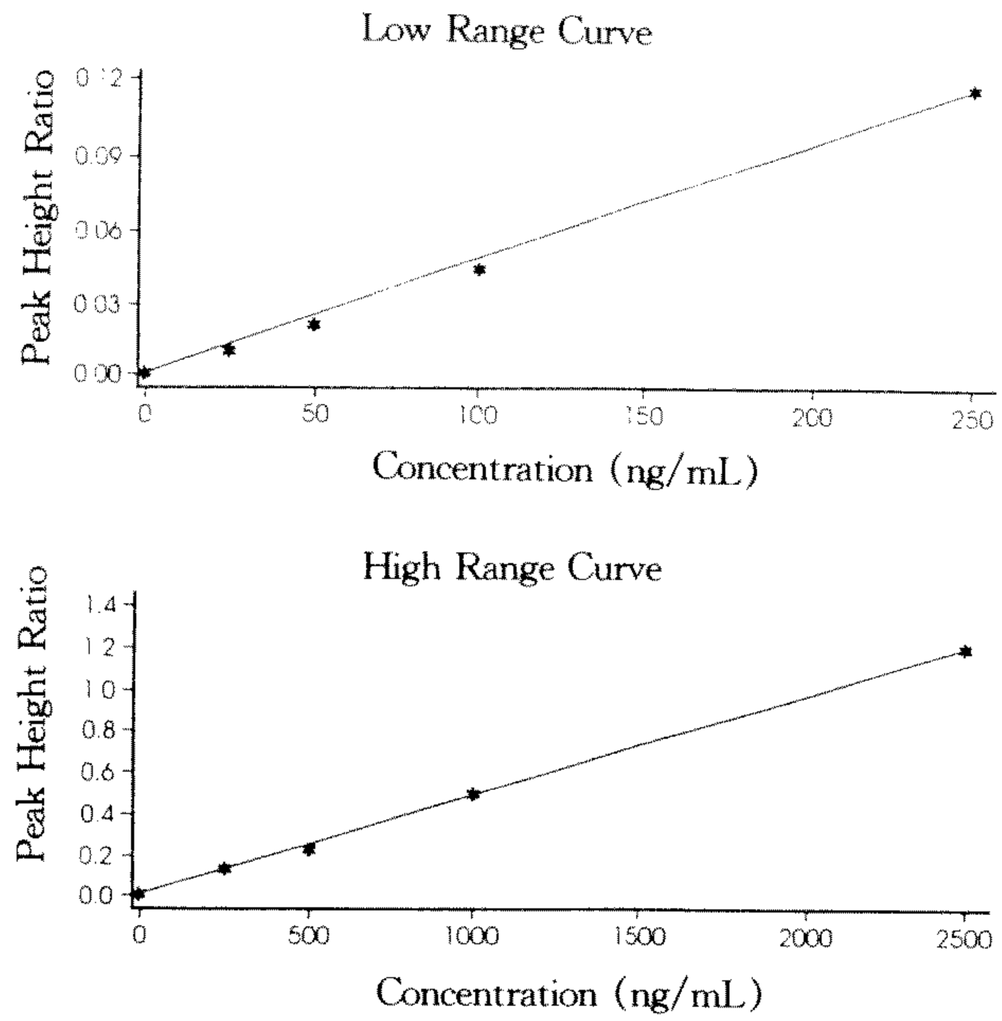


Figure 4. Calibration curves for S enantiomer in dog plasma.

of liver, NADPH (1 mM) and an NADPH regenerating system. The reactants were incubated at 37℃ for one hour. The samples were extracted and analyzed by HPLC for the R and S enantiomer concentrations.

RESULTS AND DISCUSSION

Specificity and Linearity

Typical chromatograms of blank and spiked dog plasma in Figure 2 show that R and S enantiomers were well resolved. The approximate retention time of the S enantiomer was 10.2 minutes while that of R was 11.7 minutes. Further, no endogenous compounds appear to interfere with their quantitation.

The peak height ratios of the R and S enantiomers (relative to internal standard) were linear over the concentration range of 25 to 2500 ng/ml. The calibration standards were divided into a low (25 ng to 250 ng/ml) and a high (100 ng to 2500 ng/ml) concentration range in order to obtain a more precise fit of the linear regression lines (Figure 3 and Figure 4). Correlation coefficients (r values) of all calibration curves were 0.9987 or greater. The limits of quantitation for both enantiomers was 25 ng/ml when 1 ml of plasma was extracted.

Precision and Accuracy

The inter-assay precision of this method, as measured by the percent relative standard deviations at all concentration levels for the two enantiomers on three separate days, was less than 9.5% (Table 1 and Table 2). Similarly, the accuracy of this method, as measured by the percent relative recovery on three separate days, ranged from 93.9% to 103.4% (Table 1 and Table 2).

Method Application to In Vitro and In Vivo Samples

Plasma samples obtained from dogs dosed with the R or the S enantiomer by intravenous or oral route were analyzed for the presence of the two enantiomers (Figure 5). The assay results show that the R and S enantiomers' plasma profiles were not significantly different following I. V. administration (Figure 6). Similarly, the plasma profiles of the two enantiomers were not significantly different following oral administration (Figure 7). Data from this study also indicate that there were no appreciable levels of inversion occurring following oral or I. V. administration of either the R or the S enantiomer. The results from the *in vitro* liver incubation study also show that the inversion be-

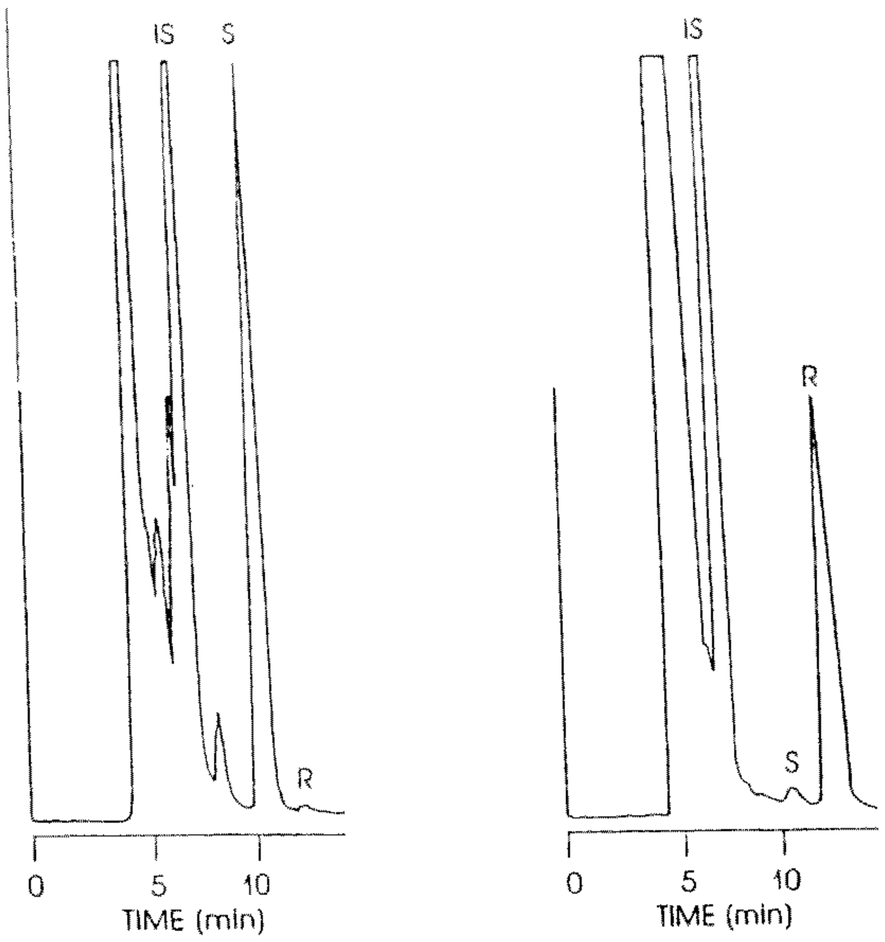


Figure 5. Chromatograms of plasma samples obtained 0.5 hours after I.V.administration of 2.5 mg/kg of the "S"(left panel) or "R"(right panel) ML-1035 enantiomers to dogs.

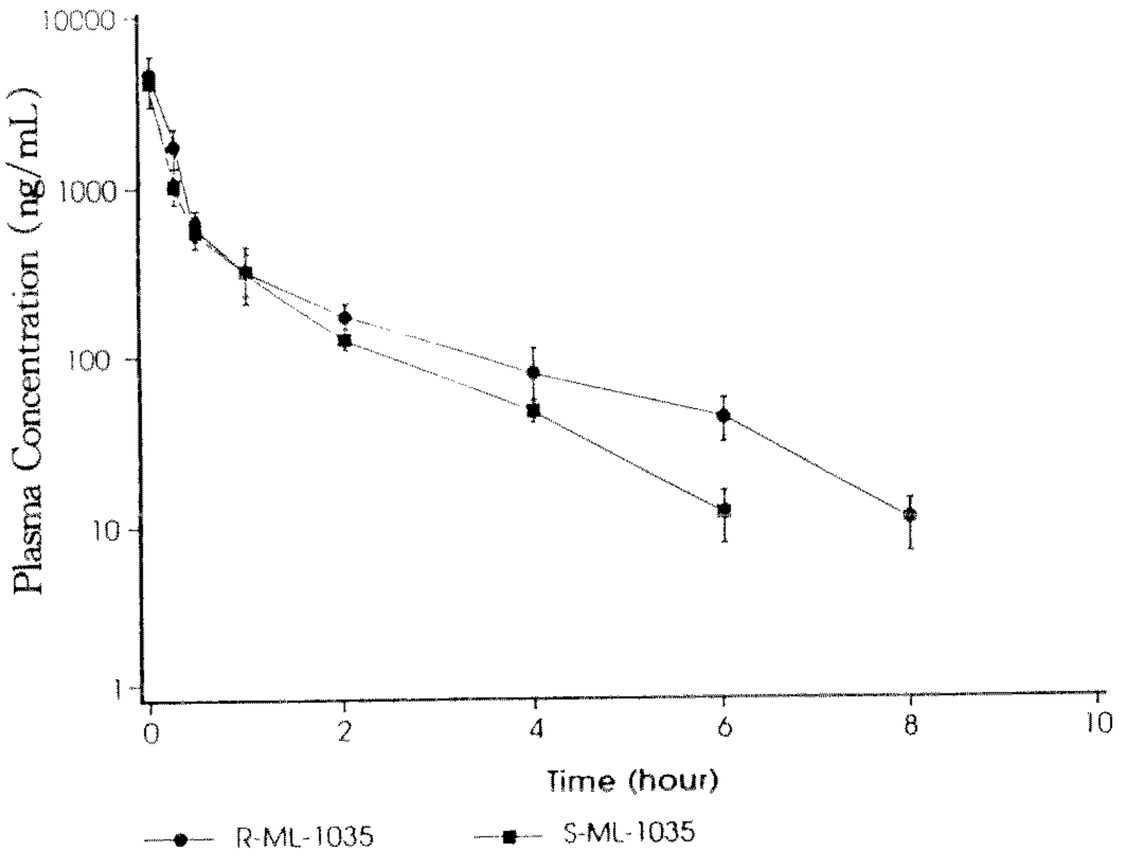


Figure 6. Plasma concentration of R and S ML-1035 in dogs after I.V.dosing of each enantiomer at 2.5 mg/kg.

tweeen the two enantiomers was not significant (Table 3).

Conclusion

A chiral HPLC method has been developed and validated for the determination of R- and S-enantiomers of ML-1035 in plasma. The method is sensitive, selective and reproducible, with a minimum quantifiable concentration of 25 ng/ml for both R- and S-enantiomers. The utility of the method was established by analysing plasma samples from dogs treated with R- or S-enantiomers and no significant differences between the plasma disposition profiles of the samples were found. In vitro and in vivo study results showed no significant degree of inversion between the R- and S-enantiomers.

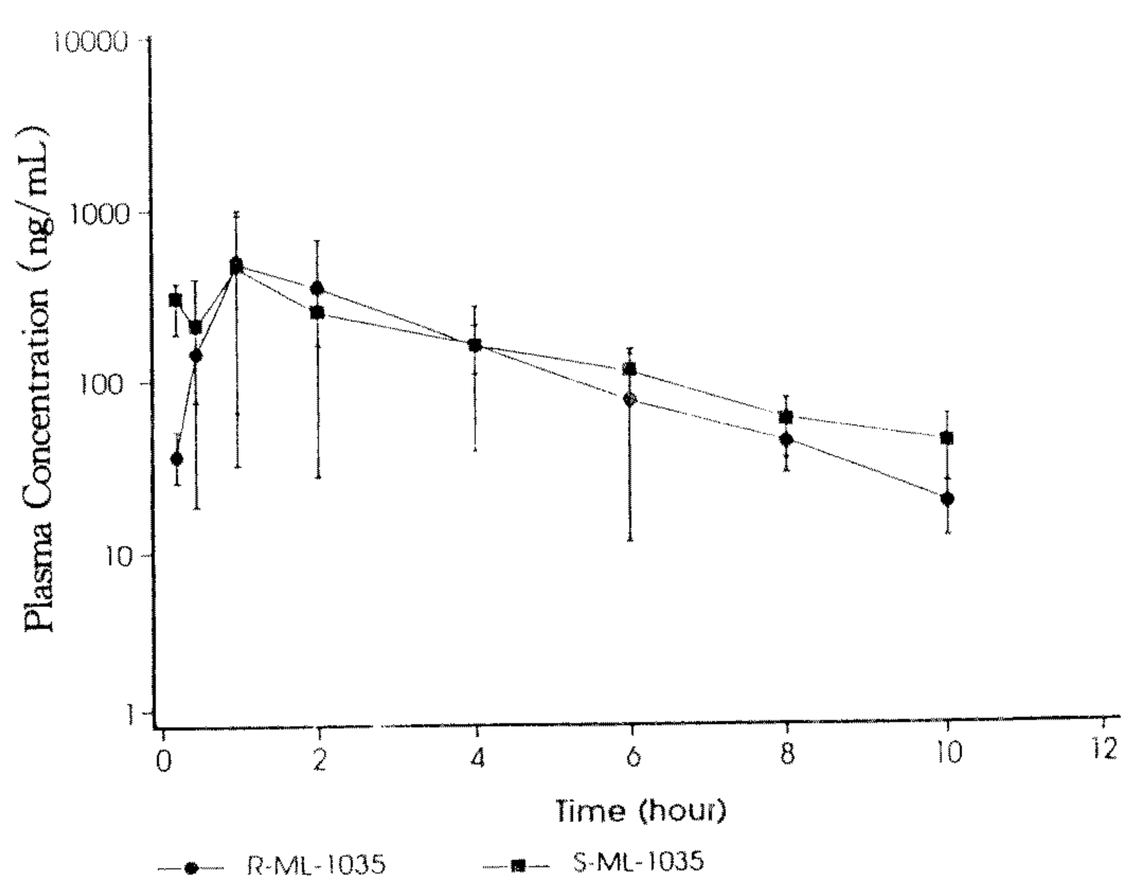


Figure 7. Plasma concentration of R and S ML-1035 in dogs after oral dosing of each enantiomer at 20 mg/kg.

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Table 1 Inter-Assay* Precision and Accuracy of R Enantiomer in Dog Plasma

Predicted Concentration (ng/ml)	Number of Replicates**	Observed Concentration*** (ng/ml) Mean ± SD	CV (%)	Relative Recovery (%)
0	3	0	--	--
25	3	25.63 ± 1.53	6.0	102.5
50	3	47.99 ± 1.59	3.3	96.0
100	3	103.4 ± 9.8	9.5	103.4
250	3	249.0 ± 3.8	1.5	99.6
500	3	484.3 ± 30.6	6.3	96.9
1000	3	964.1 ± 36.3	3.8	96.4
2500	3	2523 ± 15.3	0.6	100.9

* r values ranged from 0.9987 to 1.000, slope-mean value—0.0004
** Three sets of calibration curves containing duplicate standards were run on separate days.
The calibration standards were divided into:
low range curve (25-250 ng/ml)and
high range curve (250-2500 ng/ml)
*** The observed concentration data for 0-250 ng/ml was obtained from low range curve and, for 500-2500 ng/ml data from high range curve.

Table 2. Inter-Assay* Precision and Accuracy of R Enantiomer in Dog Plasma

Predicted Concentration (ng/ml)	Number of Replicates**	Observed Concentration*** (ng/ml) Mean ± SD	CV (%)	Relative Recovery (%)
0	3	0	--	--
25	3	25.81 ± 1.82	7.1	103.2
50	3	48.10 ± 3.75	7.8	96.2
100	3	95.99 ± 0.89	0.9	96.0
250	3	251.9 ± 0.83	0.3	100.8
500	3	496.6 ± 25.7	5.2	93.9
1000	3	995.9 ± 73.5	7.4	99.6
2500	3	2508 ± 26.6	1.1	100.3

* r values ranged from 0.9987 to 0.9998, slope-mean value-0.0005
** Three sets of calibration curves containing duplicate standards were run on separate days.
The calibration standards were divided into:
low range curve (25-250 ng/ml)
high range curve (250-2500 ng/ml)
*** The observed concentration data for 0-250 ng/ml was obtained from low range curve and, for 500-2500 ng/ml data from high range curve.

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Table 3. Stereochemical Outcome of in Vitro Incubation of R or S Enantiomers of ML－1035 in Rat and Dog Liver 10,100 × g Supernatant

Species	Substrate Stereo-Chem-istry	Drug Concentration (μg/ml)				% Inversion*
		Initial		Final		
		S	R	S	R	
Rat	S	32.4±3.6	0.2±0.3	15.6±1.1	2.68±0.7	7.7
	R	1.6±0.3	32.5±2.3	0.4±0.2	31.2±2.8	NA
Dog	S	40.8±2.5	-	34.7±2.1	2.5±0.9	6.1
	R	1.5±0.2	39.0±2.4	1.0±0.3	37.24±0.5	NA

*

$$\%INVERSION\ \textbf{of R to S} = \frac{Final\ S\ Conc.-Initial\ S\ Conc.}{Initial\ R\ Conc.} \times 100$$

$$\%INVERSION\ \textbf{of S to R} = \frac{Final\ R\ Conc.-Initial\ R\ Conc.}{Initial\ S\ Conc.} \times 100$$

NA-Not applicable.

以光學活性高效液相屬析儀分析血液中 ML-1035 對掌異構物

黃金楷， ARUN K. MANDAGERE

摘　要

ML-1035 爲類似於 mefoclopromide, 作用於胃腸道之消化劑, 其結構中具有不對稱中心, 本文敘述使用光學活性高效液相層析法分析其 R 及 S 對掌異構物之方法。血液樣本在鹼性條件下以二氯乙烷粹取, 粹取液抽乾後溶於層析分離使用之移動相。層析使用 Chiralcel OD 管柱, 以含 1% TEA 之正甲烷:絶對酒精 = 1:1(V/V) 爲移動相, 層析物以螢光偵測器(激發波長 310mm, 發射波長 350mm)偵測之。

此分析方法對 ML-1035 對掌異構物在 25 至 2500ng/ml 濃度範圍內具有線性關係, 其相關係數大於 0.9987, 不同次分析間之精確度在 ±9.5% 之間, 準確度則介於理論值之 93.9% 至 103.4% 之間。實驗狗以口服或靜脈注射 ML-1035 後, 以此方法可得到良好的分析效果, 此方法並用於試驗偵測 R 及 S 對掌異構物之轉位反應。資料顯示該對掌異構物無論在體內或體外試驗中均無因代謝而產生明顯之轉位反應。