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Validation of a reversed-phase HPLC method for directly quantifying the enantiomers of MDL 74 156, the primary metabolite of dolasetron mesylate, in human plasma[1]

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Abstract

A direct chiral HPLC method has been developed and validated to quantitate the enantiomers of MDL 74 156, the primary metabolite of dolasetron mesylate, in human plasma over the concentration range 1.70–340 ng ml^{-1}. Dolasetron mesylate is a 5-HT_3 receptor antagonist that is currently being developed as an antiemetic. Both enantiomers of MDL 74 156 and the internal standard (granisetron) were first extracted from alkanized plasma using methyl *t*-butyl ether. The analytes were then back-extracted into formic acid, separated on a ovomucoid-bonded HPLC column, and detected by native fluorescence (excitation wavelength of 274 nm and emission wavelength of 345 nm). The completed validation demonstrated the method to be accurate, precise, and specific for the direct quantitation of MDL 74 156 enantiomers in human plasma. This procedure has been used on a routine basis to quantify the relative concentrations of each enantiomer of MDL 74 156 in both oral and intravenous pharmacokinetic studies of dolasetron mesylate in normal human volunteers.

Keywords: Direct chiral reversed-phase HPLC; Dolasetron mesylate; MDL 74 156; Plasma

1. Introduction

Dolasetron mesylate (Anzemet® or MDL 73 147EF) is a serotonin receptor (5-HT_3) antagonist that is currently being developed as an antiemetic to counteract the nausea and vomiting that often accompanies chemotherapy treatment [1,2]. Once administered this compound is rapidly metabolized to its alcohol form [3,4], MDL 74 156, which has been shown to be 50 times more active than dolasetron mesylate in 5-HT_3 receptor binding assays [5]. The structures of dolasetron mesylate and MDL 74 156 are shown in Fig. 1. MDL 74 156 has an asymmetric carbon at position 3, and the (+) enantiomer, MDL 73 405, has been shown to be at least three times more potent than the (−) enantiomer, MDL 73 349 [6]. Previously, an analytical procedure was developed to

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quantify the enantiomers of MDL 74 156 in human urine using HPLC on an ovomucoid (OVM) column with UV detection [7]. However, the limit of quantification and ruggedness of this assay were not sufficient to develop a similar method in human plasma that was needed to support several pivotal dolasetron mesylate pharmacokinetic studies. This paper describes the development and validation of a reversed-phase HPLC fluorescence assay for the direct enantiomeric separation and quantitation of MDL 74 156 in human plasma over a concentration range of 1.70–340 ng ml^{-1}.

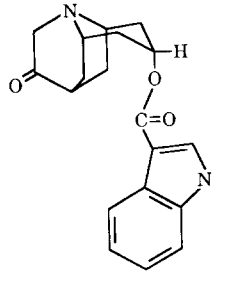
2. Experimental

2.1. Materials and reagents

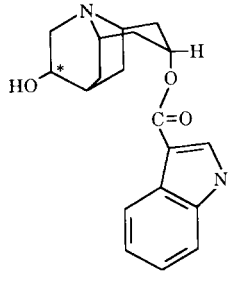
Reference standards of dolasetron mesylate, each enantiomer of MDL 74 156, and a 1.0 mg ml^{-1} stock solution of the internal standard (IS) granisetron were obtained from Hoechst Marion Roussel Inc. (Cincinnati, OH, and Strasbourg, France). HPLC-grade acetonitrile and methyl *t*-butyl ether were purchased from Burdick and Jackson (Muskegon, MI). All other reagents were of analytical grade and were purchased from Mallinckrodt (Paris, KY). Deionized water was purified through a Nanopure II system from Barnstead (Dubuque, IA). Human EDTA plasma was obtained from Biological Specialty Corp. (Colmar, PA).

2.2. Instrumentation

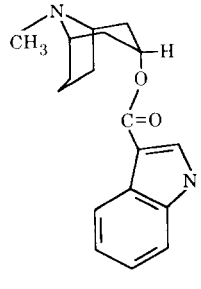
The HPLC system consisted of a Waters (Milford, MA) model 510 HPLC pump coupled to an Ultron ES-OVM (Mac Mod Analytical, Chadds Fort, PA) column followed by a Shimadzu (Acton, MA) RF10A fluorescence detector. Two SSI (State College, PA) model LP-21 low pulse dampeners were connected in series between the pump and autosampler to minimize fluctuations in the chromatographic baselines. The analytical and guard chiral OVM columns were protein-based chiral columns that were 150 mm × 4.6 mm i.d. and 10 × 4.0 mm i.d. respectively. A Waters model 715 Ultra WISP autoinjector was used for automated injection of the plasma extracts. Samples were vortexed on an American Scientific Products (McGaw Park, IL) model MT-51 variable touch mixer. A Boekel Industries (Philadelphia, PA) Orbitron rotating mixer was used for sample extraction, and the samples were spun down in an MSE (Houston, TX) Mistral 3000i benchtop centrifuge. A Beckman (Palo Alto, CA) PeakPro chromatography system was used to acquire the raw data and calculate the resulting peak heights. Data regression was performed on a SAS computer program that was written and validated internally at Hoechst Marion Roussel Inc.



Dolasetron mesylate



MDL 74,156



Granisetron

Fig. 1. Chemical structures of dolasetron mesylate, MDL 74 156 and granisetron (IS). The asterisk denotes the chiral center on MDL 74 156.

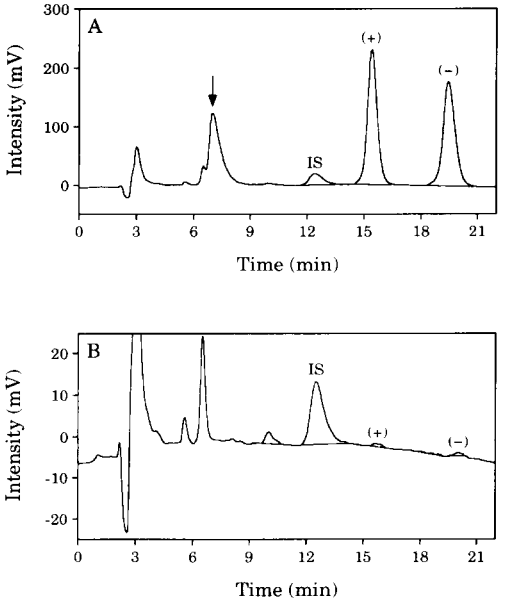


Fig. 2. Typical HPLC separations of high and low extracted plasma calibration standards. (A) Chromatogram of a 340 ng ml^{-1} standard curve sample containing both enantiomers of MDL 74 156, dolasetron mesylate, and the IS extracted from plasma. The arrow designates the retention time of dolasetron mesylate (340 ng ml^{-1}). (B) Chromatogram of a 1.70 ng ml^{-1} standard curve sample containing both enantiomers of MDL 74 156 extracted from plasma.

2.3. HPLC conditions

The HPLC pump was operated in the isocratic mode at a flow rate of 1.0 ml min^{-1} at ambient room temperature. The mobile phase consisted of acetonitrile–ammonium acetate (25 mM, pH 7.2* with ammonium hydroxide) (16:84, v/v). Minor adjustments in acetonitrile content ($\approx$ 2–3%) and pH values ($\approx$ 0.2–0.3 pH units) were necessary due to the inherent variability of the OVM columns (described in detail in Section 3). The mobile phase was filtered through 0.2 μm nylon filters and degassed for at least 5 min by helium sparging. The fluorescence detector was operated at an excitation wavelength of 274 nm and an emission wavelength of 345 nm (native fluorescence wavelength maxima of MDL 74 156). The detector was also operated with the highest sensitivity setting, a midrange gain setting, and a 5 s time constant.

2.4. Standard solution preparation

Two primary stock solutions (17.0 and 1.70 μg ml^{-1}) containing dolasetron mesylate and both the (+) and (−) enantiomers of MDL 74 156 were prepared in acetonitrile–water (50:50, v/v) and stored frozen at −20°C. These stock solutions were diluted with acetonitrile–water (5:95, v/v) to form working standards containing 17.0, 34.0, 85.0, 425, 850, 1700, 2550, and 3400 ng ml^{-1} of dolasetron mesylate and both enantiomers of MDL 74 156, and these solutions were stored at 4°C. On the individual days of analysis, plasma calibration standards containing 1.70, 3.40, 8.50, 42.5, 85.0, 170, 255, and 340 ng ml^{-1} of dolasetron mesylate and both enantiomers were prepared by adding 100 μl of the appropriate working standard to 1.0 ml of human EDTA plasma. A 10 ng ml^{-1} stock solution of granisetron (IS) in acetonitrile–water (5:95, v/v) was prepared from the 1 mg ml^{-1} stock IS solution and stored at 4°C.

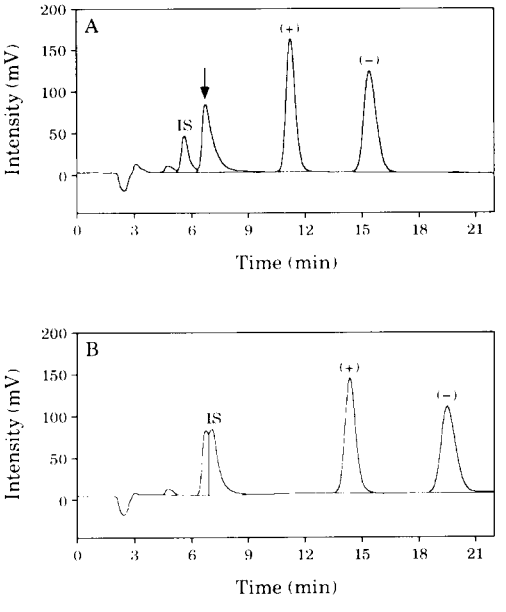


Fig. 3. Chromatographic variability of the OVM HPLC columns. The identical nonextracted solution containing 170 ng ml^{-1} of both enantiomers of MDL 74 156 and 1.0 ng ml^{-1} of the IS in 50 mM formic acid was injected onto both columns with the same mobile phase. The arrow designates the retention time of dolasetron mesylate (170 ng ml^{-1}).

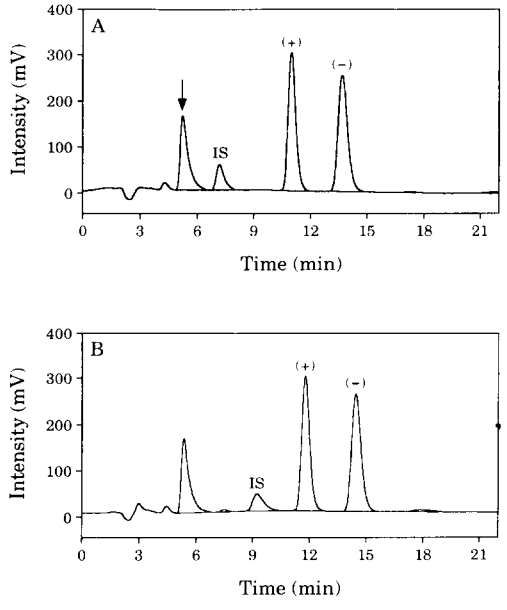


Fig. 4. Increased retention times of the analytes and IS after multiple injections of the plasma extracts. (A) The first injection of a nonextracted standard solution containing 340 ng ml^{-1} of each enantiomer of MDL 74 156 and 1.0 ng ml^{-1} of IS in 50 mM formic acid after optimizing this new OVM column. The arrow designates the retention time fo dolasetron mesylate (340 ng ml^{-1}). (B) The identical nonextracted standard was injected after 400 injections of the extracted plasma samples were made on the same column shown in (A).

2.5. Plasma quality control standard preparation

Plasma quality control (QC) samples containing 340, 170, 8.50, 3.40, and 1.70 ng ml^{-1} of dolasetron mesylate and both enantiomers of MDL 74 156 were prepared from the primary stock solutions. These QC samples were aliquoted and stored frozen in glass tubes at −20°C until analyzed.

2.6. Extraction procedures

A 100 μl aliquot of 5.0 M citric acid was added to each 1 ml of thawed EDTA plasma (matrix blanks, standard calibration, quality control, and subject samples). This step was necessary to prevent auto-oxidation of dolasetron mesylate as described previously [8]. Every plasma sample (except matrix blanks) was spiked with 100 μl of the 10 ng ml^{-1} IS solution and then vortexed. To all of the tubes, 1.0 ml of 2.0 M sodium carbonate was added followed by 7.0 ml of methyl *t*-butyl ether and 1.5 g of solid potassium chloride. After capping with Teflon-coated caps, the tubes were put on an Orbitron mixer for 20 min and spun down for 20 min at 3500 rev min^{-1}. The tubes were subsequently put in a −70°C freezer on a slant for 20 min and then placed upright on a bed of dry ice for ≈5 min. The organic layer from each tube (7.5 ml) was decanted into separate 15 ml glass screwtop centrifuge tubes each containing 500 μl of 50 mM formic acid. These tubes were capped, mixed for 15 min on an Orbitron, and centrifuged for 15 min at 3500 rev min^{-1}. The organic layer from each tube was removed by aspiration, and 350 μl of the aqueous layer was transferred into separate autosampler vials. Sequentially, 250 μl aliquots of the plasma extracts were injected onto the HPLC chiral column.

2.7. Assay validation

This method was validated over the concentration range 1.70–340 ng ml^{-1} in human plasma for each enantiomer of MDL 74 156. Calibration curves for each batch analyzed were defined by two replicates of eight freshly prepared calibration standards. Approximately 30 replicates of each of the five quality control standards were assayed over the course of three separate days. The concentration of each enantiomer of MDL 74 156 in the quality control samples was calculated by interpolation from the line of best fit for calibration standards assayed simultaneously with the quality controls. The lines of best fit for calibration standards were calculated by weighted $(1/x)$ quadratic least-squares regression based on analyte to IS peak height ratios.

3. Results and discussion

3.1. Chromatography

Although several chiral separation techniques (both direct and indirect) were investigated to resolve the enantiomers of MDL 74 156, the protein-bonded OVM column was found to be the

Table 1
Extraction recoveries of MDL 74 156 enantiomers and IS from human plasma

Drug	Conc. (ng ml^{-1})	Recovery (%)	RSD[a] (%)	n[b]
(+) MDL 74 156	17.0	79.5	11.4	6
	340	81.9	9.2	6
(−) MDL 74 156	17.0	78.9	12.5	6
	340	80.5	8.5	6
IS	1.0	75.3	8.8	6

[a] RSD = relative standard deviation.
[b] n = number of determinations.

most effective. A typical chromatogram of the completely resolved enantiomers of MDL 74 156 at 340 ng ml^{-1} is shown in Fig. 2A along with a low level chromatogram in Fig. 2B. The average resolution ratio (R_s) between the two enantiomers was 3.9 and the average enantiomeric separation

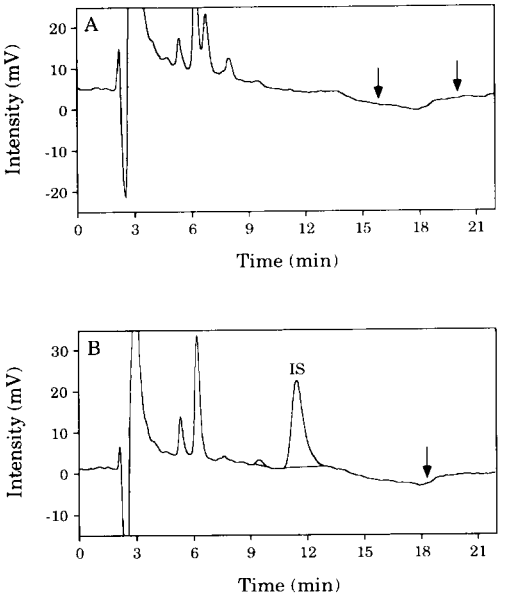


Fig. 5. Typical HPLC separation of extracted blank human plasma and zero level plasma samples. (A) Chromatogam of an extracted blank plasma sample with the arrows designating the retention times of both enantiomers of MDL 74 156. (B) Chromatogram of a zero level plasma calibration standard containing only the IS (1.0 ng ml^{-1}). The arrow designates an unusual baseline depression that was apparent on some of the OVM columns.

(a) factor was 1.3. In addition to the IS and both of the enantiomers, there is also a peak that corresponds to the parent compound, dolasetron mesylate (Fig. 2A). Unfortunately, due to its poor chromatographic peak shape and the presence of

Table 2
Intra- and inter-batch precision and accuracy of (+) MDL 74 156 quality controls

Batch	n[a]	Conc. (+) MDL 74 156 (ng ml^{-1})		RSD[b] (%)	RE[c] (%)
		Added	Measured		
1	9	1.70	1.68	12.9	−1.2
	9	3.40	3.10	7.2	−8.8
	9	8.50	7.64	5.8	−10.1
	9	170	159	4.8	−6.5
	9	340	312	3.9	−8.2
2	14	1.70	1.83	13.6	7.6
	14	3.40	3.42	7.7	0.6
	14	8.50	8.59	5.3	1.1
	14	170	177	5.2	4.1
	13	340	329	7.1	−3.2
3	13	1.70	1.76	12.5	3.5
	14	3.40	3.50	13.6	2.9
	14	8.50	9.02	6.7	6.1
	13	170	166	4.8	−2.4
	14	340	322	4.2	−5.3
Overall	36	1.70	1.75	12.7	2.9
	37	3.40	3.34	11.6	−1.8
	37	8.50	8.47	9.3	−0.4
	36	170	169	6.5	−0.6
	36	340	322	5.7	−5.3

[a] n = number of determinations.
[b] RSD = relative standard deviation.
[c] RE = relative error.

Table 3
Intra- and inter-batch precision and accuracy of (−) MDL 74 156 quality controls

Batch	n[a]	Conc. (−) MDL 74 156 (ng ml^{-1})		RSD[b] (%)	RE[c] (%)
		Added	Measured		
1	9	1.70	1.63	9.8	−4.1
	8	3.40	3.31	8.3	−2.6
	9	8.50	7.54	7.4	−11.3
	9	170	156	4.9	−8.2
	9	340	309	3.5	−9.1
2	13	1.70	1.83	6.4	7.6
	12	3.40	3.58	9.7	5.3
	13	8.50	8.45	10.1	−0.6
	14	170	175	10.1	2.9
	13	340	343	7.8	0.9
3	12	1.70	1.73	13.7	1.8
	13	3.40	3.25	12.7	−4.4
	14	8.50	7.96	8.8	−6.4
	13	170	162	5.9	−4.7
	14	340	321	3.9	−5.6
Overall	34	1.70	1.73	10.7	1.8
	33	3.40	3.37	11.3	−0.9
	36	8.50	7.96	8.8	−6.4
	36	170	165	9.0	−2.9
	36	340	326	7.1	−4.1

[a] n = number of determinations.
[b] RSD = relative standard deviation.
[c] RE = relative error.

an interfering peak, dolasetron mesylate could not be quantified to the desired level (1.0 ng ml^{-1}) and thus was disregarded in all of the subsequent analyses.

Even though the OVM columns were able to reproducibly separate the enantiomers of MDL 74 156 from each other and the IS, dramatic differences were found between columns that had been packed by the manufacturer on the same day with the identical lot of OVM material. As shown in Fig. 3, this column-to-column variability resulted in shifts in the retention times of the enantiomers by as much as 4 min. To overcome these differences between the OVM columns, the acetonitrile concentrations and pH of the mobile phase had to be optimized from column to column. Once the chromatographic separation had been optimized, the retention time of the (+) enantiomer of MDL 74 156 ranged from 11.5–15.5 min while that of the (−) enantiomer ranged from 16.0–21.0 min and that of the IS ranged from 8–13 min.

Multiple injections of the extracted plasma samples caused both enantiomers of MDL 74 156 and the IS to be retained on the column longer. As shown in Fig. 4, this migration in retention times continued with each injection to a point where the IS could not be completely resolved from the (+) enantiomer of MDL 74 156. The injections of the back-extracted plasma samples in 50 mM formic acid (pH 3) may have slowly changed the retentive characteristics of the ovomucoid protein immobilized on the HPLC column, which gradually pushed the analytes and the IS out to longer retention times. These columns have a recommended pH range between 3.0 and 7.5 and repeated injections of large volumes of formic acid could have changed the conformational structure of the OVM protein over time.

Alternatively, modification of the retentive behavior of the OVM column may have been caused by nonspecific adsorption of highly retained components from the plasma extracts as was described previously [9]. These changes were found to be irreversible since extensive washing with acetonitrile [10] did not regenerate the columns to their original performance characteristics. Despite these changes, the retention time shifts did not pose a problem for the validation or overall ruggedness of this method. Numerous injections of the plasma extracts just decreased the useful lifetime of an individual OVM column to $\approx$ 400 injections.

3.2. Extraction recovery

The extraction efficiency was determined to be 80% for each enantiomer and 75% for the IS, based on a direct comparison with nonextracted standards (Table 1). Additional studies demonstrated that during the extraction procedure there was no interconversion of the two enantiomers of MDL 74 156 (data not shown).

Table 4
Stability of MDL 74 156 enantiomers in human plasma[a]

Stability experiment	Conc. (+) MDL 74 156 (ng ml^{-1})		Conc. (−) MDL 74 156 (ng ml^{-1})	
	17.0	340	17.0	340.0
Freeze/thaw ($n = 10$)				
Initial	16.7 (2.9)	329 (3.2)	16.4 (3.6)	324 (3.9)
1st cycle	16.7 (2.4)	336 (3.6)	16.8 (4.5)	330 (4.5)
As % of initial	100	102	102	102
2nd cycle	16.3 (3.0)	328 (2.5)	16.2 (4.7)	319 (3.2)
As % of initial	98	100	99	98
On-system ($n = 5$)				
Initial	18.2 (8.5)	374 (10.2)	18.1 (10.3)	371 (10.7)
48 h	18.4 (9.5)	358 (7.1)	18.2 (10.3)	348 (7.0)
As % of initial	101	96	101	94
Long term ($n = 5$)				
Initial	17.3 (9.4)	342 (3.5)	17.7 (4.4)	340 (3.3)
195 days	17.5 (6.7)	339 (6.7)	17.5 (4.4)	340 (6.2)
As % of initial	101	99	99	100
365 days	18.1 (6.3)	339 (7.4)	18.7 (9.1)	336 (4.7)
As % of initial	105	99	106	99

[a] RSDs (%) are indicated in parentheses.

3.3. Specificity

During the development of this method no interferences were found from either the parent drug (dolasetron mesylate) or other known metabolites of MDL 74 156 [11,12]. Chromatograms of a plasma blank (one of the six different lots of plasma that were screened) and zero level standard (IS only) samples indicated no interferences from the matrix (Figs. 5A and 5B respectively). A characteristic baseline depression occurred at $\approx$ 19 min on some of the new OVM columns that diminished in magnitude and increased in retention time as the number of injections on the column increased (Fig. 5B). This unexplainable depression did not interfere with the quantitation of these analytes but reintegrations were often required at the lower concentrations of both of the enantiomers of MDL 74 156.

3.4. Validation

The assay was validated over the concentration range 1.70–340 ng ml^{-1} for each enantiomer of MDL 74 156. Two replicates of eight freshly prepared calibration standards (1.70–340 ng ml^{-1}) and a minimum of nine replicates of five quality control standards (14 on days 2 and 3) were assayed on three separate days in groups called batches. The line of best fit for calibration standards was calculated for each day of analysis by weighted ($1/x$) quadratic least-squares regression based on peak height ratios. The r^2 values were greater than 0.99 in all of the batches for both enantiomers of MDL 74 156. Accuracy and precision of the calibration standards were determined using the back-calculated values from the calculated regression line for that day. The accuracy of the intra-batch calibration standards varied from 91–112% for the (+) enantiomer and 95–109%

for the (−) enantiomer. The inter-batch mean accuracy of the calibration standards ranged from 98–103% for the (+) enantiomer and 95–104% for the (−) enantiomer.

Quality control samples at five concentration levels (1.70–340 ng ml^{-1}) were assayed on three separate days. Tables 2 and 3 summarize both intra- and inter-batch validation statistics for the (+) and (−) enantiomers of MDL 74 156 respectively. The inter-batch accuracy ranged from 95–103% for the (+) enantiomer and 94–102% for the (−) enantiomer. The inter-batch precision (%RSD) was 5.7–12.7% for the (+) enantiomer and 7.1–11.3% for the (−) enantiomer. The intra-batch accuracy and precision for the quality control samples ranged from 90–108% and from 3.9–13.6% respectively for the (+) enantiomer. For the (−) enantiomer of MDL 74 156 the intra-batch accuracy and precision for the quality control samples ranged from 89–108% and from 3.5–13.7% respectively.

3.5. Stability

Further experiments verified that both enantiomers of MDL 74 156 were stable throughout the sample storage and chromatography procedures. Table 4 summarizes the results from these stability experiments. After 12 months of being stored at −20°C in glass tubes, at least 99% of both enantiomers was recovered when compared to freshly prepared samples. Both enantiomers of MDL 74 156 were also stable through at least two cycles of freezing and thawing when compared to freshly prepared samples. The plasma extracts were also found to be stable in the HPLC injection solvent for at least 48 h at room temperature. Finally, interconversion of the two enantiomers did not occur over the 12 month incubation time or through the two freeze/thaw cycles (data not shown).

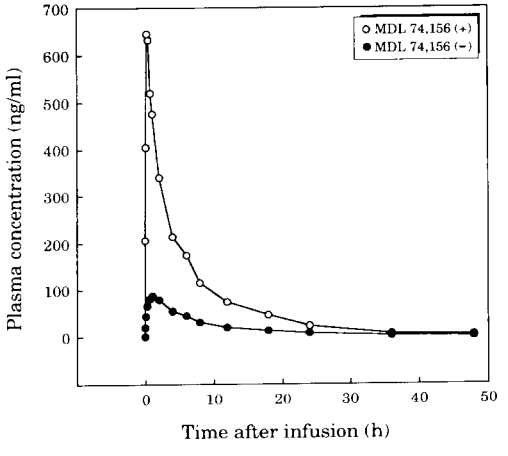


Fig. 6. Typical pharmacokinetic profile of both enantiomers of MDL 74 156 in an individual normal human volunteer who was administered a 200 mg intravenous solution of dolasetron mesylate.

4. Conclusions

An accurate, precise, and specific method for the direct quantitation of the enantiomers of MDL 74 156 in citrated EDTA human plasma was developed and validated over the concentration range 1.70–340 ng ml^{-1}. This method was relatively simple and rapid, allowing the analysis of at least 96 samples per batch. This method has been used to quantitate the relative concentrations of the enantiomers of MDL 74 156 in both oral and intravenous dolasetron mesylate pharmacokinetic studies in normal human volunteers (Fig. 6). Preliminary reports of these studies have been reported previously [13].

Acknowledgements

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References

[1] M. Galvan, M.W. Gittos and M. Fatmi, Drugs Future, 18 (1993) 506–509.
[2] R.C. Miller, M. Galvan and M.W. Gittos, Drug Dev. Res., 28 (1993) 87–93.
[3] H. Boxembaum, T. Gillespie, K. Heck and W. Hahne, Biopharm. Drug Dispos., 13 (1992) 693–701.
[4] H. Boxembaum, T. Gillespie, K. Heck and W. Hahne, Biopharm. Drug Dispos., 14 (1993) 131–141.

[5] P.H. Boeijinga, M. Galvan, B.M. Baron, M.W. Dudly, B.W. Siegel and A.L. Stone, Eur. J. Pharmacol., 219 (1992) 9–13.
[6] J. Dow and C. Berg, Chirality, 7 (1995) 342–348.
[7] M. Biguad, L. Elands, P.R. Kastner, R.A. Bohnke, L.W. Emmert and M. Galvan, Drug Dev. Res., 34 (1995) 289–296.
[8] T.A. Gillespie, J.A. Eckstein, P. Nardella and J.E. Coutant, J. Pharm. Biomed. Anal., 11 (1993) 955–962.
[9] K.Y. Chan, D.A. Dusterhoft and T.-M. Chen, J. Chromatogr. Biomed. Appl., 656 (1994) 359–365.
[10] J. Haginaka, T. Murashima, H. Fujima and H. Wada, J. Chromatogr. Biomed. Appl., 620 (1993) 199–204.
[11] P. Sanwald, N.D. Huebert and K.D. Haegele, J. Chromatogr. Biomed. Appl., 661 (1994) 101–107.
[12] M.K. Reith, G.D. Sproles and L.K. Cheng, Drug Metab. Dispos., 23 (1995) 806–812.
[13] Y.S. Choo, D.C. Dimmitt, J.S. McElvain, M.A. Castles, T. Arumugham, V.J. Vandiver, V.O. Bhargava, M.E. Eller, W.F. Hahne and S.J. Weir, Pharm. Res., 12 (1995) S388.