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Journal of Chromatography B, 796 (2003) 165–172
JOURNAL OF CHROMATOGRAPHY B
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Reliable and specific high-performance liquid chromatographic method for simultaneous determination of loratadine and its metabolite in human plasma

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Received 18 March 2003; received in revised form 8 July 2003; accepted 12 August 2003

Abstract

A high-performance liquid chromatographic (HPLC) method with fluorescence detection has been developed for the simultaneous determination of loratadine (L) and its metabolite, descarboethoxyloratadine (DCL), in human plasma. Following a two-step liquid–liquid extraction with toluene, the analytes were separated using a gradient mobile phase consisting of methanol–acetonitrile–phosphate buffer. The linearity for L and DCL was within the concentration range of 0.5–16 ng/ml. The coefficient of variation of intra- and inter-day assay was <8.3%, with accuracy ranging from 98.3 to 105.7%. The lower limit of quantification was 0.5 ng/ml for both L and DCL. This method has been demonstrated to be reliable, and is an improvement over existing methods due to its capability for determining L and DCL simultaneously in a single chromatographic run.

Keywords: Loratadine; Descarboethoxyloratadine

1. Introduction

Loratadine, ethyl-4-(8-chloro-5,6-dihydro-11H-benzo[5,6]cyclohepta[1,2*b*]pydridin-11-ylidene)-1-piperidine carboxylate, is a long-acting antihistamine with selective H1 receptor antagonistic activity. Loratadine (L) undergoes extensive first-pass metabolism in the liver, forming an active metabolite, descarboethoxyloratadine (DCL) (Fig. 1) [1].

Several methods such as radioimmunoassay (RIA) [2], gas chromatograph–mass spectrometry (GC–MS) [3], and high-performance liquid chromatography (HPLC) [4] have been described for the determination of L alone in human plasma. However, the determination of both L and DCL is more useful for clinical pharmacokinetic studies since DCL possesses similar pharmacological activity as L. Although separate analytical techniques, i.e. RIA for L and HPLC for DCL, have been utilized in the previous pharmacokinetic studies [2,5,6], such an approach is inconvenient.

Recently, the use of the same assay method for both compounds has been reported. These include a

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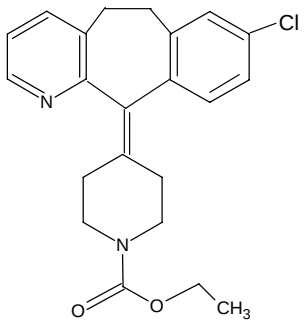
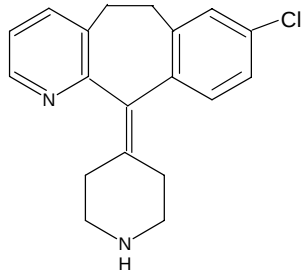
doi:10.1016/j.jchromb.2003.08.023



Loratadine (L)



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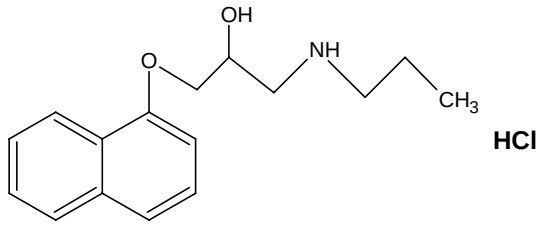

Propranolol hydrochloride (IS)

Fig. 1. Chemical structures of: loratadine (L), descarbo-ethoxyloratadine (DCL) and propranolol hydrochloride (IS).

HPLC [7], GC [8], and LC–MS [9] method. The HPLC method requires two separate HPLC systems for L and DCL, respectively [7]. Its need for two specifically synthesized internal standards also limits the application of this method. The GC method is highly sensitive and specific, however, it involves complicated sample preparation procedures as well as two chromatographic runs for L and DCL [8]. Although a LC–MS method provides the simultaneous determination of L and DCL with one chromatographic run [9], its sample preparation, which included several extraction and back-extraction steps, is quite laborious and time consuming. The use of external standards is one additional limitation of this method.

This paper describes a reliable and specific HPLC method for the simultaneous determination of L and DCL in human plasma. The method employs a gradient mobile phase together with a relatively simple liquid–liquid extraction procedure, to determine both L and DCL in a single chromatographic run.

2. Experimental

2.1. Reagents and chemicals

L and DCL were obtained from Sequoie Research Products Ltd. (Oxford, UK). Propranolol hydrochloride (internal standard (IS)) was obtained from Sigma (St. Louis, MO, USA). The purity of L, DCL and propranolol hydrochloride were all >99%. Methanol, acetonitrile, toluene, potassium dihydrogen phosphate, sodium hydroxide and hydrochloride acid were purchased from BDH (Dorset, UK). Except for methanol and acetonitrile which are of HPLC grade, all others are of analytical grade.

2.2. Preparation of standard solution

Stock solutions of L and DCL were prepared by dissolving the appropriate amount of each powder in a solution containing methanol and 0.05 M HCl (20:80

(v/v)), to yield a concentration of 1 mg/ml. Working solutions of L and DCL were prepared by appropriate dilution.

Standard solution of IS was prepared by dissolving the appropriate amount of propranolol hydrochloride powder in a solution containing methanol and 0.05 M HCl (20:80 (v/v)), to yield a concentration of 7.5 μg/ml.

2.3. Apparatus and chromatographic conditions

The HPLC system consisted of a Waters Alliance® 2690 separation module, Waters 474 fluorescence detector and Millennium32 chromatography management system (Waters, Milford, MA, USA). Chromatographic separation was achieved on a Waters Symmetry C_{18} column (150 mm × 3.9 mm, 5 μm; Waters, Milford, MA, USA) preceded with an Alltech 0.2 μm filter column. The mobile phase, consisting of a mixture of methanol, acetonitrile and 0.05 M potassium dihydrogen phosphate buffer (pH 2.0), was run using a linear gradient elution program as shown in Table 1. The flow rate was set at 1.2 ml/min, and the total run time was 20 min. The column was maintained at 35 °C and the auto-sampler at 5 °C. Fluorescence detection was performed at an excitation wavelength of 290 nm and an emission wavelength of 480 nm.

2.4. Sample preparation

To a 1 ml aliquot of plasma in a 10 ml conical centrifuge tube, 20 μl of IS solution (7.5 μg/ml of propranolol hydrochloride solution) and 200 μl of 1 M sodium hydroxide solution were added. After vortex mixing, 3 ml of toluene was added. The mixture was then shaken for 20 min and centrifuged at 2000 × *g* for 10 min. The aqueous phase was frozen at −20 °C and the organic phase decanted into a new 10 ml conical glass tube. After thawing, the aqueous phase was extracted again with 3 ml of toluene using the same procedure as above. The organic phase from two extractions were combined, and evaporated to dryness in a water bath at 50 °C under a stream of nitrogen. The residue was reconstituted with a 200 μl solution containing methanol and 0.05 M HCl (20:80 (v/v)), vortex mixed briefly, and transferred to a clean HPLC auto-sampler vial. A 100 μl of aliquot was subsequently injected for HPLC analysis.

Table 1
Gradient elution program for HPLC separation of L, DCL and IS

Time (min)	Solvent (%)[a]			Flow rate (ml/min)
	A	B	C	
0	5	14	81	1.2
5.5	3	24	73	1.2
10	3	40	57	1.2
14[b]	5	14	81	1.2

[a] Solvent: A, methanol; B, acetonitrile; C, phosphate buffer (pH 2.0).

[b] Composition of mobile phase was changed linearly. At the end of each run (i.e. 14 min), the column was left to equilibrate at the starting mobile phase composition for additional 6 min. Thus, the total run time is 20 min.

2.5. Validation of the assay method

2.5.1. Specificity

Chromatographic interference from endogenous plasma components was investigated using pooled blank plasma samples as well as samples from healthy subjects who participated in a clinical pharmacokinetic study of L.

2.5.2. Linearity

Plasma samples were spiked in five replicates at concentrations of 0.5, 1, 2, 4, 8, and 16 ng/ml. The samples were assayed using the method described above. The standard calibration curves for L and DCL were constructed using the analyte/IS peak–area ratios versus the nominal concentrations of the analytes. Linear least-squares regression analysis with weighting factor of $1/x$ was performed to assess the linearity as well as to generate the standard calibration equation:

$$y = ax + b$$

where y is the peak–area ratio, x the concentration, a the slope and b the intercept of the regression line.

2.5.3. Recovery

Spiked plasma samples were prepared in triplicate at concentrations of 0.75 and 7.5 ng/ml, and assayed as described above. Recovery (extraction efficacy) was calculated by comparing the peak area of the extracted sample to that of the unextracted standard solution containing the same concentration.

2.5.4. Precision and accuracy

The precision and accuracy of this method were evaluated using quality control samples at concentrations of 0.5, 1.5, 5 and 15 ng/ml. For intra-day assay precision and accuracy, six replicates of quality control samples at each concentration, a total of 24, were assayed all at once within a day. The inter-day assay precision and accuracy was determined by analyzing the quality control samples on three different days. Six replicates at each concentration were assayed per day.

2.5.5. Sensitivity

The lower limit of quantification (LLOQ) was determined for both L and DCL, based on the criteria that: (1) the analyte response at LLOQ is five times of baseline noise; (2) the analyte response at LLOQ can be determined with sufficient precision and accuracy, i.e. precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was determined as the lowest concentration which gives a signal-to-noise ratio of 3 for L and DCL.

2.5.6. Stability of analytes

Stability samples were prepared at concentrations of 1.5 and 15 ng/ml. For freeze–thaw stability testing, the concentrations of the samples (in triplicate at each concentration) after three freeze–thaw cycles were determined and compared to the freshly prepared samples. The auto-sampler stability was evaluated by comparing the extracted plasma samples that were injected immediately with those injected after placing in the auto-sampler at 5 °C for 24 or 48 h. Nine replicates of samples at each concentration were extracted using the same procedure as described above. Of the extracted samples, three were injected immediately, three injected after placing in the auto-sampler for 24 h, and three injected after placing in the auto-sampler for 48 h.

3. Results

3.1. Separation and specificity

Fig. 2 shows the representative chromatograms of a blank plasma, plasma samples spiked with L and DCL at LLOQ (0.5 ng/ml) and at 4 ng/ml, and an authentic plasma sample obtained from a healthy subject following an oral 20 mg dose of L. The analytes were well separated using the present chromatographic conditions. The retention times were 3.4 min for DCL, 8.6 min for IS, and 11.2 min for L. No interfering peaks from the endogenous plasma components were observed at the retention time of each analyte or IS.

3.2. Calibration and linearity

The calibration curves were linear over the concentration range of 0.5–16 ng/ml for both L and DCL. The mean linear regression equations of standard curves are:

$$\text{L}: \quad y = 0.0694(\pm 0.0025)x - 0.0091(\pm 0.0011), \quad r = 0.9976 \pm 0.0017$$

$$\text{DCL}: \quad y = 0.0559(\pm 0.0025)x - 0.0028(\pm 0.0010), \quad r = 0.9961 \pm 0.0021$$

3.3. Recovery

For plasma concentrations at 0.75 and 7.5 ng/ml, the mean recovery of L were 80.4 and 81.8%, respectively; and the mean recovery of DCL were 67.2 and 69.8%, respectively. The recovery of IS averaged 77.5%.

3.4. Precision and accuracy

The precision and accuracy for measurement of L and DCL are summarized in Tables 2 and 3. The mean coefficients of variation (CVs (%)) for intra- and inter-day assay were all <8.3%. The accuracy ranged from 98.3 to 106%.

3.5. Sensitivity

The lower limit of quantification (LLOQ) was 0.5 ng/ml for both L and DCL. The limit of detection (LOD) was 0.25 ng/ml for L and DCL.

3.6. Stability

The freeze–thaw stability results show that both L and DCL are stable for at least three freeze–thaw cycles. The mean bias was from −7.1 to 6.3% for L and −3.6 to 4.1% for DCL after three freeze–thaw

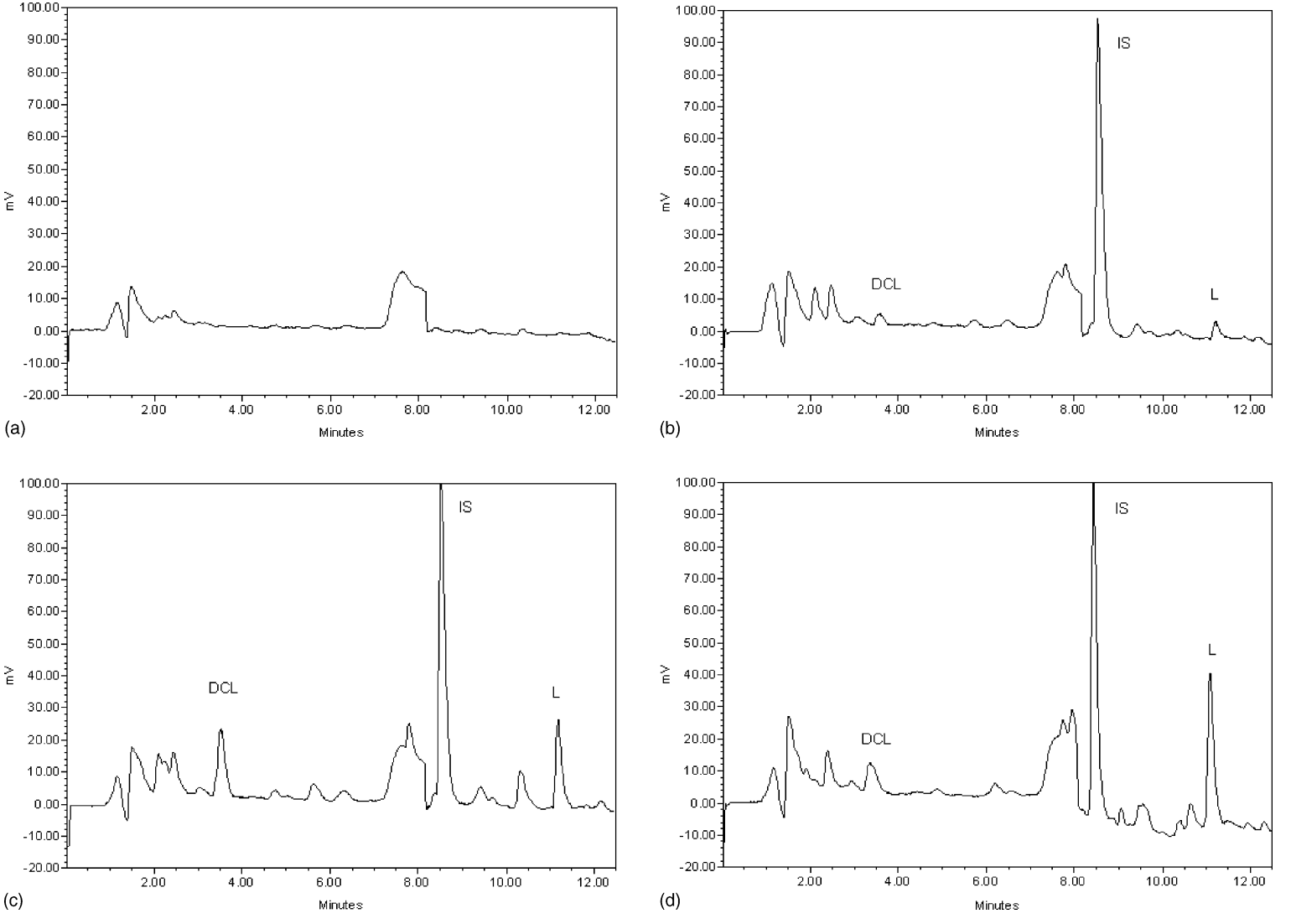

Fig. 2. Chromatograms of: (a) a blank plasma; (b) spiked plasma sample at LLOQ (0.5 ng/ml L, 0.5 ng/ml DCL and 150 ng/ml IS); (c) plasma sample spiked with 4 ng/ml L, 4 ng/ml DCL and 150 ng/ml IS; (d) plasma sample from a healthy subject following a 20 mg oral dose of L, the plasma concentration was determined to be 14.4 ng/ml for L and 3.1 ng/ml for DCL.

Table 2
Intra-day assay precision and accuracy for L and DCL

Nominal concentration (ng/ml)	Determined concentration[a] (Mean ± S.D. (ng/ml))	R.S.D. (%)	Accuracy[b] (%)
L			
0.5	0.52 ± 0.03	5.39	104
1.5	1.54 ± 0.07	4.53	102
5	4.99 ± 0.21	4.26	99.8
15	15.4 ± 0.78	5.06	103
DCL			
0.5	0.49 ± 0.04	8.28	98.3
1.5	1.50 ± 0.06	4.21	100
5	5.01 ± 0.29	5.76	100
15	15.8 ± 0.64	4.08	105

[a] $n = 6$.
[b] Calculated as (mean determined concentration/nominal concentration) × 100%.

cycles. The difference in the back-calculated concentration from time 0 (samples are injected immediately after extraction) to time 24 h (samples are placed in auto-sampler for 24 h before injection) was <1.2% for L and <2.2% for DCL, whereas the difference between time 0 and 48 h was <0.5% for L and <4.8% for DCL. The results suggest that the processed samples are stable for 48 h at 5 °C in an auto-sampler.

3.7. Dilution

The dilution study was also conducted to assess whether the upper concentration limit (16 ng/ml) can be extended. Quality control samples (in six replicates) at concentration of 60 ng/ml was diluted by five times with blank plasma, and the assay precision and accuracy were determined in a similar manner as described in Section 2.5.4. For L, the precision was 3.3% and accuracy 99.9%. Whereas, for DCL, the precision and accuracy were 4.0 and 99.1%, respectively. The results suggest that samples whose concentrations are greater than the upper limit of the standard curve can be re-analyzed by appropriate dilution.

Table 3
Inter-day assay precision and accuracy for L and DCL

Nominal concentration (ng/ml)	Determined concentration[a] (Mean ± S.D. (ng/ml))	R.S.D. (%)	Accuracy[b] (%)
L			
0.5	0.53 ± 0.03	6.49	106
1.5	1.49 ± 0.10	7.03	99.3
5	5.07 ± 0.15	3.06	101
15	15.5 ± 0.74	4.78	103
DCL			
0.5	0.51 ± 0.03	6.12	103
1.5	1.50 ± 0.05	3.37	99.8
5	5.10 ± 0.29	5.61	102
15	15.6 ± 0.52	3.32	104

[a] $n = 3$ days with six replicates per day.
[b] Calculated as (mean determined concentration/nominal concentration) × 100%.

3.8. Application to clinical study

The method has been applied to a clinical pharmacokinetic study of loratadine in 16 healthy subjects. The concentrations were generally detectable in the study subjects up to 24 h for L and 48 h for DCL. Fig. 3 shows the representative concentration–time curves of L and DCL in one subject following a 20 mg oral dose of L under fasting condition. The absorption of L and subsequent formation of DCL is rapid, with peak concentrations occurring at 1 h for L and 1.5 h for DCL. The peak concentrations of L and DCL are 10.7 and 7.7 ng/ml, respectively. The apparent oral clearance (CL/F) of L is estimated to be 6.7 L/(h kg) in this subject (Genotype analysis reveals that this subject is a homozygous carrier of intermediate metabolizer associated alleles, 2D6*10/*10). These parameter values are in general agreement with those reported previously [2,5]. The observed rapid absorption of L and

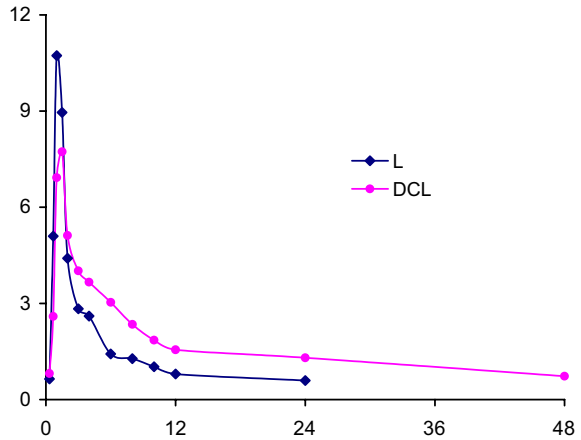

Fig. 3. Plasma concentration–time profiles of L and DCL in a healthy subject following a 20 mg oral dose of L.

formation of DCL in our study are also consistent with previous study which demonstrated that the onset of action of L is within 1 h [10].

4. Discussion

Simultaneous determination of L and DCL is difficult due to their differences in polarity and pK_a values. Previous reported analytical methods are all very time consuming and are not suitable for large number of samples. In this study, we developed a reliable and efficient HPLC technique to determine both L and DCL in human plasma.

The major changes include the chromatographic conditions, especially the mobile phase components and the gradient elution program, which were optimized to reduce the run time and obtain good separation of analytes. Although L and DCL could be separated using an isocratic mobile phase of acetonitrile and phosphate buffer (20:80), the total run time was as long as 60 min (the retention time of DCL and L was 8 and 50 min, respectively). The use of gradient elution of mobile phase, i.e. increasing the proportion of acetonitrile gradually from time 0 to 5.5 min and then 5.5 to 10 min, significantly reduced the retention time of both analytes and thus shortened the whole run time. A high proportion of methanol could cause prolonged retention time as well as reduced fluorescence response for both analytes, however, the addition of small amount of methanol (3–5%) was found to improve the mixing of the mobile phase effectively. Also, the choice of pH of the mobile phase was found to be crucial. L and DCL only exhibited strong fluorescence response at pH <4.0. In this study, a pH value of 2.0 seemed to be optimal for the separation and detection of L and DCL.

Contrary to the observation by Sutherland et al. [9], we found toluene can extract both L and DCL simultaneously from plasma. Repeating the extraction with toluene improved the extraction efficacy significantly (especially for DCL), yielding a satisfactory recovery of 80.4–81.8% for L and 67.2–69.8% for DCL. Thus, by combining a new mobile phase with gradient elution program as well as a two-step liquid–liquid extraction procedure, this HPLC method is capable of simultaneous determination of L and DCL in a single chromatographic run.

Instead of using specifically synthesized internal or external standards as described in previous methods [7,9], we used a readily available chemical, propranolol hydrochloride, to serve as the internal standard. The recovery of propranolol hydrochloride from plasma averaged 77.5% and was satisfactory.

With the aid of fluorescence detection, plasma concentrations as low as 0.5 ng/ml (LLOQ) for both L and DCL could be reliably detected with no analytical interference from the plasma. Although better sensitivities (LOQ 0.1 ng/ml) had been obtained in some other methods [8,9], an LLOQ of 0.5 ng/ml was found to be sufficient for determining the real plasma samples from our pharmacokinetic study of L in healthy subjects.

Our method has a relatively short turnover time (20 min) and is suitable for clinical pharmacokinetic studies. Although this turnover time is not as short as a previous LC–MS method [9] which only need 6.4 min for one run, the LC–MS machine, however, is quite expensive and is not readily available in most clinical research laboratories. Thus, our present assay technique provides a reliable and acceptable method for many laboratories with HPLC availability.

5. Conclusion

A reliable and specific method for the simultaneous determination of L and DCL in human plasma has been developed, using a HPLC with fluorescence detection. The method is suitable for clinical pharmacokinetic studies, and is an improvement over existing methods due to its capability for determining both L and DCL in a single chromatographic run.

Acknowledgements

This study was supported in part by Grant ITS/174/00 from the Innovation and Technology Commission of the Government of Hong Kong SAR.

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