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Stereospecific determinations of (±)-DU-124884 and its metabolites (±)-KC-9048 in human plasma by liquid chromatography

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Abstract

(+)-DU-124884 is a 5-HT_1-like receptor agonist under investigation for drug development. A sensitive, stereospecific LC method was developed for the analysis of (+)-DU-124884, its optical isomer (−)-DU-124884 and their *N*-dealkylated metabolites, (±)-KC-9048, in human plasma. A plasma sample was treated with triethylamine in methanol and the proteins were precipitated by acetonitrile. The supernatant was evaporated to dryness under nitrogen. The analytes and internal standard (acebutolol) formed diastereomers with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate immediately. The diastereomers formed were extracted into diethyl ether. They were completely resolved from each other and from matrix peaks on a Microsorb silica column with a mobile phase of methanol–chloroform–hexane (8:12:80, v/v/v) in a run time of 26 min. Detection was by fluorescence with excitation wavelength at 320 nm and emission wavelength at 440 nm. The linearity range is 0.1–200 ng ml^{-1} ($r > 0.99$). The limit of quantitation is 0.1 ng ml^{-1} and the detection limit is 0.02 ng ml^{-1} (signal-to-noise ratio = 3). The interday precision and accuracy of quality control samples were 5.5–7.6% RSD (relative standard deviation) and 0 to +4% bias for (+)-DU-124884, 5.5–7.9% RSD and 0 to +4% bias for (−)-DU-124884, 4.5–6.5% RSD and −7 to 0% bias for (+)-KC-9048 and 4.5–7.5% RSD and −7 to 0% bias for (−)-KC-9048. Consistent recovery from different lots of human plasma, parallelism of the method, stabilities of on-system, reinjection, bench-top, freeze–thaw cycles and sample storage were established.

Keywords: (±)-DU-124884 and metabolites; Enantiomers; Stereospecific liquid chromatographic analysis; Human plasma

1. Introduction

(*R*)(+)-DU-124884 {(+)-3-methylaminomethyl-3,4,5,6-tetrahydro-6-oxo-1*H*-azepino-

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[5,4,3-*cd*]indole} is a new 5-HT_1-like receptor agonist under investigation for drug development. A sensitive stereospecific LC method is needed to monitor (+)-DU-124884 which is intended for clinical testing. The method should also quantify its optical isomer (−)-DU-124884, which can be potentially formed from (+)-DU-124884 in vivo and their *N*-dealkylated metabolites, (+)-KC-9048 and (−)-KC-9048. The structures of (±)-DU-124884 and (±)-KC-9048 are shown in Fig. 1.

The purpose of this study was to develop and validate a sensitive (0.1 ng ml^{-1}) and reliable LC method for the quantitation of (±)-DU-124884 and (±)-KC-9048 enantiomers in human plasma. This method should also be able to measure the concentration of the (−)-enantiomers in the presence of their (+)-isomers at a disproportionately higher concentration. A normal-phase LC method using precolumn derivatization with the chiral reagent (*S*)-(+)-1-(naphthyl)ethyl isocyanate (S-NEIC) was developed. As low as 0.1 ng ml^{-1} of each enantiomer can be quantified using a 0.5 ml plasma volume. The development and validation of this method are reported here.

2. Experimental

2.1. Materials and reagents

Racemic (±)-DU-124884 hydrochloride and (±)-KC-9048 were obtained from Solvay Pharmaceutical (Marietta, GA, USA). The potencies (w/w) expressed as free base were 86.2% and 92.9% for (±)-DU-124884 and (±)-KC-9048, respectively, with corresponding chromatographic purities of 99.9% and 99.6%. (+)-DU-124884 hydrochloride, (−)-DU-124884 hydrochloride, (+)-KC-9048 and (−)-KC-9048 were also obtained from Solvay Pharmaceutical. Their chromatographic purities were greater than 98%. Racemic acebutolol, the internal standard (I.S.) and triethylamine (TEA) of analytical grade were purchased from Sigma (St. Louis, MO, USA). (*S*)-(+)-1-(1-Naphthyl)ethyl isocyanate (S-NEIC), +99%, was purchased from Aldrich (Milkwaukee, WI, USA). Chloroform, dichloromethane, methanol, hexane, acetonitrile and diethyl ether of HPLC grade were obtained from Fisher (Fair Lawn, NJ, USA). Dehydrated ethanol of HPLC grade was purchased from Quantum (Newark, NJ, USA). All other reagents were of analytical grade from Mallinckrodt (Paris, KY, USA). Deionized water was purified with a Nanopure system from Barnstead (Dubuque, IA, USA). Control heparianized human plasma was obtained from Worldwide Biological (Cincinnati, OH, USA).

S-NEIC in chloroform (1%, w/v) was prepared and stored at −20°C. This solution was used for up to 2 months and no adverse affect on derivatization was noticed. The stability of the solution beyond 2 months was not determined. TEA in methanol (0.5%, w/v) was freshly prepared daily.

2.2. Instrumentation

The LC system consisted of a Beckman 110B solvent-delivery module (Berkeley, CA, USA), a Shimadzu SIL-9A autosampler (Kyoto, Japan), a Shimadzu RF-551 fluorescence detector set at an excitation wavelength of 320 nm and an emission wavelength of 440 nm and a VG Mutichrom data system for VAX/VMS (Manchester, UK). In order to prevent solvent evaporation and potential degradation of the derivatized compounds, the autosampler tray was cooled at 10°C by a Brinkman RM6 cooling system (Westbury, NY, USA). The autosampler needle washing solvent was methanol. The flow rate was 1.0 ml min^{-1} and the run time was set at 26 min. The analytical column, a Microsorb silica column of 5 μm, 25 cm × 0.46 cm i.d., was obtained from Rainin (Woburn, MA, USA). The guard column was a Microsorb silica column of 5 μm, 3 cm × 0.46

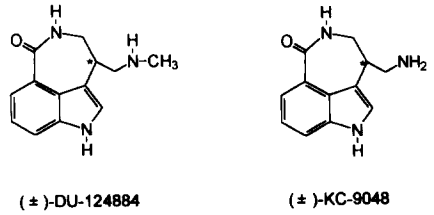

Fig. 1. Structures of (±)-DU-124884 and (±)-KC-9048. The asterisks denote chiral centers.

cm i.d., which was replaced after every 300 injections. One analytical column was used throughout the whole validation for more than 1000 injections. The mobile phase was methanol–chloroform–hexane (8:12:80, v/v/v). The mobile phase can be used for up to 2 weeks without recycling.

2.3. Extraction procedures

To 0.5 ml of plasma sample, 100 μl of methanolic I.S. solution (2000 ng ml^{-1}) and 250 μl of TEA in methanol were added. After mixing and standing for 10 min, 1 ml of acetonitrile was added to precipitate the plasma proteins. The supernatant was separated and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.5 ml of dehydrated ethanol and evaporated to dryness again. Methanol (0.5 ml) followed by 25 μl of S-NEIC in chloroform were added. After mixing, the solvents were evaporated under nitrogen. Water (0.5 ml) and 4 ml of diethyl ether were added to extract the formed diastereomers. The organic extract was evaporated to dryness under nitrogen and reconstituted in 200 μl of chloroform. A 50 μl volume was injected into the LC system.

The unextracted solutions for recovery tests were prepared by adding 50 μl of TEA in methanol to the standard solutions prepared in water instead of plasma in the same way as working standards.

Polypropylene tubes were used throughout the extraction. Glass tubes were used in some experiments to check the adsorption of the compounds on the glass.

2.4. Working standards and quality control samples

Two primary stock methanolic solutions of (±)-DU-124884 and (±)-KC-9048 were prepared from separate weighings for standard and quality control samples (QCs). One primary methanolic solution of (+)-DU-124884 and one of (+)-KC-9048 were also prepared. The stock solutions stored at −20°C were stable for at least 2 months. Working standards were prepared fresh daily by spiking 50 μl of 10-fold concentrated solutions into 0.5 ml of blank control plasma. The final concentrations of each enantiomer in plasma standards were 0.1, 0.25, 0.5, 1, 5, 10, 50, 100, 150 and 200 ng ml^{-1}. Three levels of QCs in human plasma, 0.26, 5.2 and 129 ng ml^{-1} for (+)-DU-124884 and (−)-DU-124884 and 0.28, 5.6 and 139 ng ml^{-1} for (+)-KC-9048 and (−)-KC-9048, were prepared, aliquoted and stored frozen at −20°C. One level of parallelism QCs, 258 ng ml^{-1} for (+)-DU-124884 and (−)-DU-124884 and 278 ng ml^{-1} for (+)-KC-9048 and (−)-KC-9048, were prepared and stored at −20°C. Prior to extraction, the parallelism QC was diluted two- and fourfold with a blank control plasma other than the one used to prepare the parallelism QCs.

2.5. Data treatment

Chromatograms were measured using a VG Mutichrom data system. The raw data output was acquired on a VG Chromserver and then transferred to the VAX/VMS. A weighted $1/y$ linear regression was used to determine slopes, intercepts and correlation coefficients, where y = the ratio of the compound peak height to the internal standard peak height. The resulting parameters were used to calculate concentrations:

$$\text{concentration} = [\text{ratio} - (y\text{-intercept})]/\text{slope}$$

3. Results and discussion

3.1. Development of the LC method

The aim of the study was to develop and validate a chiral LC method which will allow the assessment of pharmacokinetic profiles for the individual enantiomers and provide information on the possible in vivo conversion of (+)-DU-124884 to (−)-DU-124884 and (+)-KC-9048 to (−)-KC-9048. No method was available for the chiral or achiral separation of (±)-DU-124884 and (±)-KC-9048 or compounds of similar structure. Direct separations of the enantiomers were attempted on several commonly used chiral stationary phase with various combinations of mo-

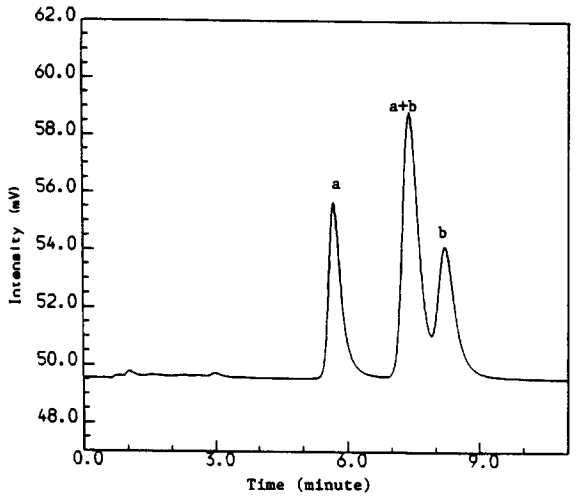

Fig. 2. Chiral separation of (±)-DU-124884 and (±)-KC-9048 on a chiral α-AGP column. Mobile phase: 0.05 M potassium phosphate buffer (pH 7.0). Column: chiral α-AGP, 5 μm, 10 cm × 0.4 cm i.d., at 23°C. Flow-rate: 0.8 ml min^{-1}. Detection: fluorescence, Ex = 320 nm and Em = 440 nm. Peak identification: a = one of the enantiomers of KC-9048; b = one of the enantiomers of DU-124884.

bile phases. Chiral separation was not achieved on β-cyclodextrin, acetyl-β-cyclodextrin, Chiralcel OD and chiral HSA. Separation of enantiomers was achieved on a chiral α-AGP column. Fig. 2 shows the separation of enantiomers at an optimized pH and ionic strength of the mobile phase. Enantiomers of KC-9048 were baseline separated whereas the separation of DU-124884 enantiomers was incomplete. We did not attempt to determine the elution order for each pair of enantiomers since the later eluting enantiomer of KC-9048 coeluted with the earlier eluting enantiomer of DU-124884 even under the optimized conditions. The desired limit of quantitation (LOQ) could not be reached because of the baseline noise under the reversed-phase conditions. Therefore, a precolumn derivatization using a chiral isocyanate reagent was pursued.

Several chiral isocyanate reagents have been reported in the literature. Chiral isocyanate reagents react with primary or secondary amine groups of the enantiomers to form urea derivatives; the formed diastereomers can be separated on an achiral stationary phase. (*S*)-(−)-1-Phenylethyl isocyanate (S-PEIC) has been used for the resolution of metoprolol [1], acebutolol and diacetolol [2]. 2,3,4,5-Tetra-*O*-acetyl-α-D-glucopyranosyl isothiocyanate (GITC) was used for the assay of bevantolol enantiomers in human plasma [3]. Diastereomers formed with S-PEIC and GITC were resolved using the reversed-phase mode, which is less desirable for the present study owing to the higher background noise. Quantitation of enantiomers of acebutolol [4], diacetolol [5], metoprolol [6], pindolol [7], sotalol [8], tertatolol [9] and tocainide [10] in biological fluids, mexiletine from microbial fermentation media [11] and enantiomeric impurities of (+)-pseudoephedrine [12] have been reported using S-NEIC as a precolumn chiral derivatization reagent. The optical isomer of S-NEIC, R-NEIC, has also been used for the quantitation of enantiomers of betaxolol [13], fluoxetine and norfluoxetine [14,15]. The diastereomers were separated on a normal-phase silica column, except for betaxolol, where a reversed-phase C-18 column was used.

It was not possible to separate the enantiomers of DU-124884 and KC-9048 after derivatization with S-NEIC on CN or C-18 columns in the reversed-phase mode, or on a CN column in the normal-phase mode. It was very interesting to note that the formed diastereomers were separated on a β-cyclodextrin column with a polar mobile phase consisting of TEA, acetic acid, methanol and acetonitrile. Fig. 3 shows the optimized separation. The mechanism of the separation is unknown. It was known that the β-cyclodextrin column was very stable in the polar mobile phase mode [16]. However, owing to the matrix interference peaks, the desired LOQ of 0.1 ng ml^{-1} for each enantiomer was not obtained. In addition, the (+)-enantiomers were eluted in front of the (−)-enantiomers, which would make it more difficult to quantify small amounts of (−)-enantiomers in the presence of excess amounts of (+)-enantiomers.

On a silica column, the presence of a chlorinated solvent in the mobile phase was necessary, otherwise, the peak shapes of the diastereomers were poor and the separation of derivatized enantiomers was not achieved. With a mobile phase of methanol–chloroform–hexane (8:12:80, v/v/v),

the retention times in minutes are 16.0 for (−)-KC-9048, 17.2 for (+)-KC-9048, 19.8 for (−)-DU-124884 and 21.5 for (+)-DU-124884. The use of a mobile phase containing dichloromethane instead of chloroform reversed the elution order of derivatives of (+)-KC-9048 and (−)-DU-124884 from that with the chloroform mobile phase. A better separation for the enantiomer pairs was obtained. Unfortunately, the peaks of (−)-DU-124884 and (+)-KC-9048 derivatives overlapped.

When the mobile phase contained more than 30% (v/v) of chloroform, the resolution of (+)-KC-9048 and (−)-DU-124884 derivatives decreased dramatically after volumes of the mobile phase had passed through the silica column. The resolution decreased from 1.3 to 0.6 in 9 h with a mobile phase of methanol–chloroform–hexane (4:36:60, v/v/v). When a freshly prepared mobile phase of the same composition was used, the resolution between derivatives of (+)-KC-9048 and (−)-DU-124884 was restored to 1.3. Investigation of this phenomenon was beyond the scope of the present study. With a mobile phase of methanol–chloroform–hexane (8:12:80, v/v/v), such a loss of resolution in time was not observed. The retention times and resolutions were constant throughout the runs. The guard column was replaced after every 300 injections. One analytical column was used for over 1000 injections and no significant loss of performance was observed.

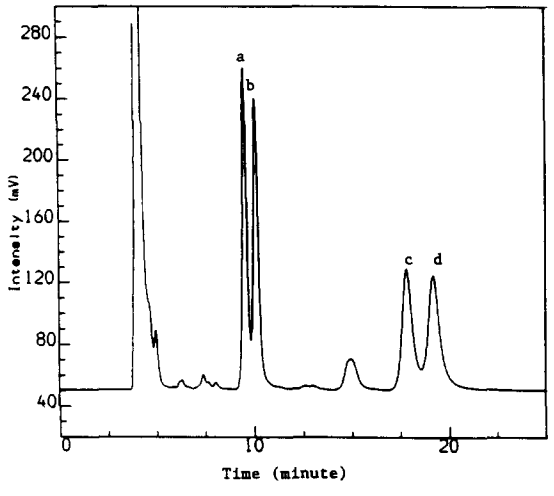

Fig. 3. Chiral separation of (±)-DU-124884 and (±)-KC-9048 on a β-cyclodextrin column after derivatization with S-NEIC. Mobile phase: triethylamine–acetic acid–methanol–acetonitrile (2.75:2.75:50:950, v/v/v/v). Column: β-cyclodextrin, 25 cm × 0.46 cm i.d., at 23°C. Flow-rate: 1.0 ml min^{-1}. Detection: fluorescence, Ex = 320 nm and Em = 440 nm. Peak identification: a = (+)-DU-124884; b = (−)-DU-124884; c = (+)-KC-9048; d = (−)-KC-9048.

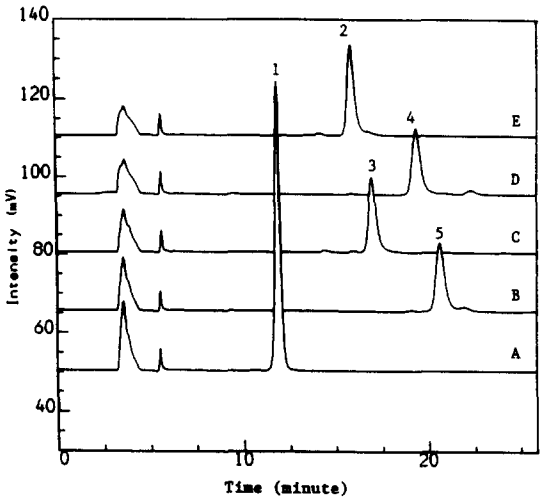

Fig. 4. Chiral separation of (A) internal standard, (B) (+)-DU-124884, (C) (+)-KC-9048, (D) (−)-DU-124884 and (E) (−)-KC-9048 on a silica column after derivatization with S-NEIC. Mobile phase: methanol–chloroform–hexane (8:12:80, v/v/v). Column: Microsorb SI, 5 μm, 25 cm × 0.46 cm i.d. at 23°C. Flow-rate: 1.0 ml min^{-1}. Detection: fluorescence, Ex = 320 nm and Em = 440 nm. Peak identification: 1 = I.S. (acebutolol); 2 = (−)-KC-9048; 3 = (+)-KC-9048; 4 = (−)-DU-124884; 5 = (+)-DU-124884.

An excellent separation of the formed diastereomers was achieved on a silica column with a mobile phase of methanol–chloroform–hexane (8:12:80, v/v/v). Fig. 4 shows the separation of derivatives of the I.S., (−)-KC-9048, (+)-KC-9048, (−)-DU-124884 and (+)-DU-124884. The diastereomers of the I.S. enantiomers after reacting with S-NEIC were coeluted as a sharp peak. The effect of column temperature on the separation was investigated. Though the plate number per column increased slightly with an elevated column temperature, the resolutions for the separation of (+)-KC-9048 and (−)-DU-124884 derivatives were slightly decreased. Therefore, the column was maintained at 23 ± 2°C for all further studies.

Fig. 5 shows chromatograms of extracted blank control plasma, blank control plasma spiked with I.S. and blank control plasma spiked with I.S. and 0.1 ng ml^{-1} each of the enantiomers. No interference was observed in all five different lots of control human plasma tested. Owing to the use of normal-phase chromatography with a mobile phase of organic solvents and to the fluorescence detection, the background noise was very low, which allowed accurate and automatic integration without manual baseline correction. The separation of each enantiomer pair is excellent even at high concentration, allowing accurate quantitation of (−)-enantiomers at low concentration in the presence of (+)-enantiomers at a disproportionately higher concentration. Fig. 6 shows a chromatogram of extracted test QC spiked with only the (+)-enantiomers. The minor amounts of (−)-enantiomers were resolved, indicating that the small amounts of the (−)-enantiomers can be precisely measured.

Six different lots of silica column were tested for the column–column variability. The column characteristics were calculated and are summarized in Table 1. The lot-to-lot column variability was such that the performance was not adversely affected by the choice of column. Excellent resolution was obtained on all the columns. The run time is less than 30 min for all the columns tested.

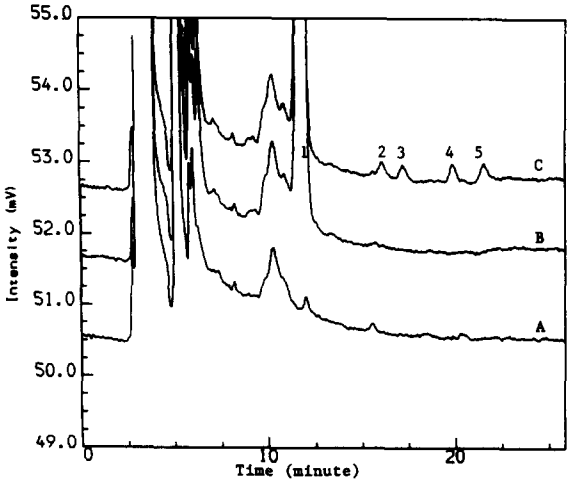

Fig. 5. Extracted (A) blank control plasma, (B) blank plasma spiked with I.S. and (C) blank control plasma spiked with I.S. and 0.1 ng ml^{-1} each of the enantiomers. See Fig. 4 for the chromatographic conditions and peak identifications.

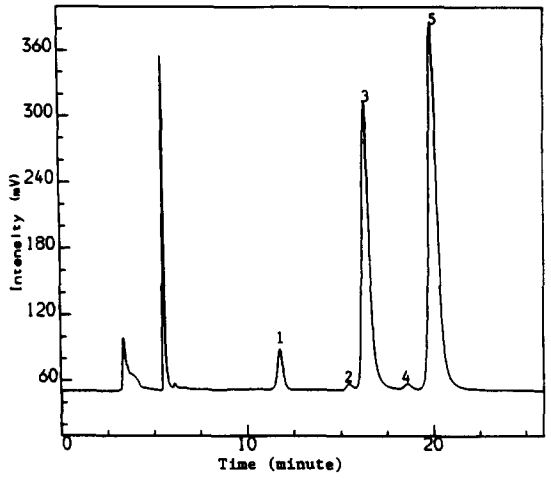

Fig. 6. Extracted plasma sample spiked only with (+)-DU-124884 and (+)-KC-9048. See Fig. 4 for the chromatographic conditions and peak identification.

3.2. Development of an extraction method

Liquid–liquid extraction of (±)-DU-124884 and (±)-KC-9048 from plasma was attempted. A plasma sample was treated with TEA and various organic solvents to extract the compounds of interest. The best extraction recovery was 72% for (±)-DU-124884 and 42% for (±)-KC-9048 using ethyl acetate as extraction solvent. Recoveries for KC-9048 enantiomers were poor and variable.

Using solid-phase extraction (SPE), better recoveries for (±)-KC-9048 were obtained on three lots of SPE C-18 cartridge (50–80%), but on the fourth lot the recovery was very poor (<10%). The lot-to-lot variabilities of the SPE cartridge prevented us from obtaining consistent results.

An extraction and derivatization procedure was then developed. A plasma sample was treated with TEA in methanol in order to release the compounds from the proteins. Fig. 7 shows the effect of the TEA concentration on the extraction efficiency. Without TEA treatment, the recoveries of both (±)-DU-124884 and (±)-KC-9048 were low. TEA released the analytes, especially (±)-KC-9048, from protein binding. A 0.25 ml volume of 0.5% (v/v) of TEA in methanol was sufficient.

Table 1
Column characteristics

Column	(+)-DU-124884		Resolution		
	Plate number per column	Peak symmetry	(−)-KC-9048 (+)-KC-9048	(+)-KC-9048 (−)-DU-124884	(−)-DU-124884 (+)-DU-124884
A	8570	1.17	1.35	2.93	1.79
B	13190	1.00	2.00	4.45	2.51
C	11880	1.00	1.75	3.39	2.23
D	13310	0.91	2.03	3.96	2.64
E	9830	1.21	1.88	4.28	2.51
F	6720	1.33	1.22	2.85	1.73

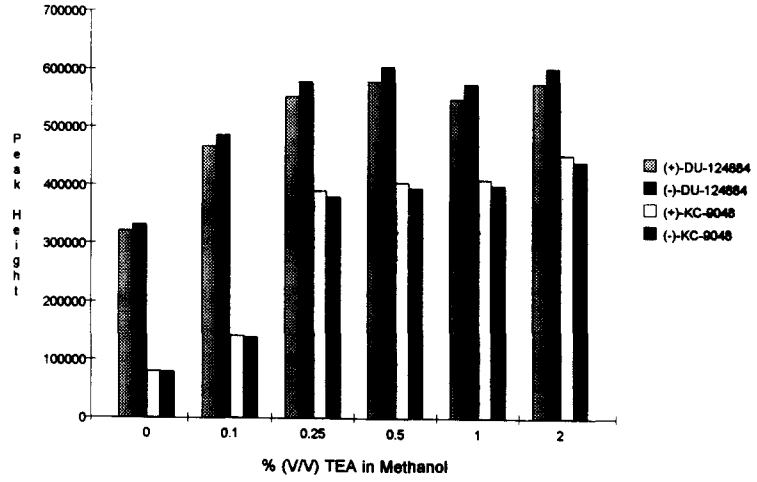

Fig. 7. Influence of the concentration of triethylamine in 0.25 ml of methanol on the extraction efficiency.

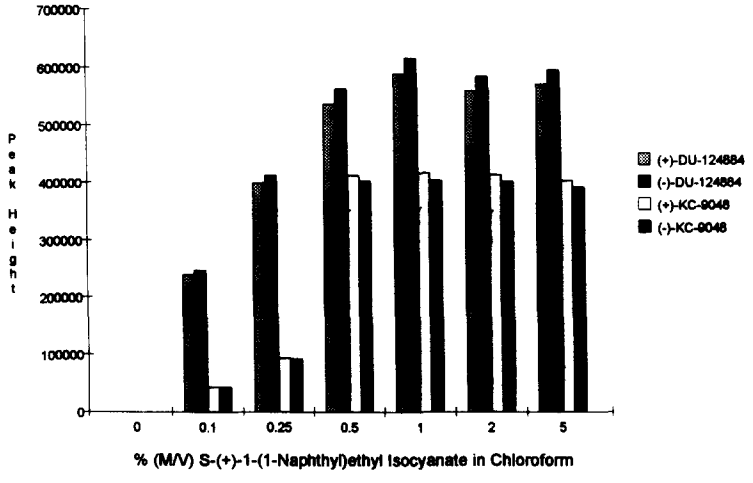

Fig. 8. Influence of the concentration of S-NEIC in 25 μl of chloroform on the derivatization efficiency.

Table 2
Recovery of (±)-DU-124884 and (±)-KC-9048 from human plasma using polypropylene and glass tubes

Compound	Parameter	Concentration (ng ml^{-1})					
		Polypropylene tubes			Glass tubes		
		0.1	5	200	0.1	5	200
(+)-DU-124884	Recovery (%)	90	89	91	79	88	96
	RSD (%)	8.0	11	11	2.4	6.9	1.4
	n	5	5	5	2	2	2
	Mean recovery (%)		90			88	
(−)-DU-124884	Recovery (%)	99	90	91	93	90	96
	RSD (%)	5.2	11	11	0.4	6.6	1.6
	n	5	5	5	2	2	2
	Mean recovery (%)		93			93	
(+)-KC-9048	Recovery (%)	99	81	79	40	62	87
	RSD (%)	9.8	11	12	4.7	10	5.3
	n	5	5	5	2	2	2
	Mean recovery (%)		87			63	
(−)-KC-9048	Recovery (%)	103	80	78	54	63	86
	RSD (%)	17	11	12	4.8	10	5.4
	n	5	5	5	2	2	2
	Mean recovery (%)		87			68	

Table 3
Intraday reproducibility of limit of quantitation (0.1 ng ml^{-1})

Parameter	(+)-DU-124884	(−)-DU-124884	(+)-KC-9048	(−)-KC-9048
Observed results	0.10	0.10	0.09	0.09
	0.10	0.09	0.10	0.12
	0.10	0.09	0.10	0.11
	0.11	0.12	0.11	0.11
	0.11	0.10	0.09	0.08
Mean	0.10	0.10	0.10	0.10
RSD (%)	5.3	12	8.4	15
RE (%) [a]	0	0	0	0

[a] RE = relative error.

The release was instantaneous upon the TEA addition. In order to assure complete release, a 10 min time period after adding TEA was used. A longer time (investigated up to 60 min) did not have any adverse effect on the extraction. The proteins were then precipitated with acetonitrile. The supernatant was evaporated to dryness under nitrogen. A second evaporation procedure after adding 0.5 ml of dehydrated alcohol was necessary to obtain consistent derivatization results by removing excess water, which reacts competitively with S-NEIC. We chose dehydrated alcohol over other solvents because it is polar enough to dissolve the residues completely and because it removes the water easily during the evaporation owing to the azeotropic effect. The residue was then dissolved in methanol. The analytes and I.S. formed diastereomers with the chiral S-NEIC. Derivatization in other organic solvents mentioned in the literature such as chloroform, dichloromethane and hexane gave lower and variable reaction yields, probably owing to the poor

Table 4
Interday precision and accuracy of (±)-DU-124884 and (±)-KC-9048 calibration standards ($n = 10$)

Compound	Parameter	Standard concentration (ng ml^{-1})										r
		0.1	0.25	0.50	1.0	5.0	10.0	50	100	150	200	
(+)-DU-124884	Mean	0.11	0.25	0.51	0.99	4.9	10.0	49	100	147	205	0.999
	RSD (%)	7.4	3.1	7.5	5.9	5.7	6.8	5.5	5.8	2.7	6.8	0.13
	RE (%)	+10	0	+2	−1	−2	0	−2	0	−2	+3	
(−)-DU-124884	Mean	0.11	0.25	0.51	0.99	4.9	10.0	49	100	148	205	0.999
	RSD (%)	7.8	4.8	7.5	5.6	5.7	6.7	5.5	5.7	2.9	6.7	0.13
	RE (%)	+10	0	+2	−1	−2	0	−2	0	−1	+3	
(+)-KC-9048	Mean	0.09	0.24	0.51	1.0	5.2	10.2	50	100	150	200	0.999
	RSD (%)	15.7	8.7	4.5	5.7	5.4	5.5	4.6	3.7	3.8	4.7	0.10
	RE (%)	−10	−4	+2	0	+4	+2	0	0	0	0	
(−)-KC-9048	Mean	0.09	0.24	0.51	1.0	5.2	10.2	50	100	150	200	0.999
	RSD (%)	12.0	9.8	5.6	5.6	5.1	5.3	4.6	3.4	3.7	4.4	0.10
	RE (%)	−10	−4	+2	0	+4	+2	0	0	0	0	

Table 5
Precision and accuracy of (±)-DU-124884 and (±)-KC-9048 quality controls

Compound	Quality control concentration (ng ml^{-1})			Compound	Quality control concentration (ng ml^{-1})		
	0.26	5.2	129		0.28	5.6	139
Interday ($n = 30$) [a]							
(+)-DU-124884				(+)-KC-9048			
Mean	0.26	5.2	134	Mean	0.26	5.6	139
RSD (%)	7.6	5.9	5.5	RSD (%)	6.5	5.4	4.5
RE (%)	0	0	+4	RE (%)	−7	0	0
(−)-DU-124884				(−)-KC-9048			
Mean	0.26	5.2	134	Mean	0.26	5.6	139
RSD (%)	7.9	6.0	5.5	RSD (%)	7.5	5.4	4.5
RE (%)	0	0	+4	RE (%)	−7	0	0
Intraday ($n = 6$) [a]							
(+)-DU-124884				(+)-KC-9048			
Mean	0.26	5.1	132	Mean	0.26	5.6	141
RSD (%)	4.8	5.7	4.1	RSD (%)	7.2	6.1	3.9
RE (%)	0	−2	+2	RE (%)	−7	0	+2
(−)-DU-124884				(−)-KC-9048			
Mean	0.27	5.1	132	Mean	0.27	5.7	142
RSD (%)	6.1	5.5	4.2	RSD (%)	9.7	5.9	3.8
RE (%)	+4	−2	+2	RE (%)	−4	+2	+2

[a] n = Number of determinations.

solubility of (±)-DU-124884 and (±)-KC-9048 in these solvents. Methanol as reaction medium has been reported for the derivatization of betaxolol enantiomers with R-NEIC [14]. The influence of the concentration of S-NEIC on the reaction is shown in Fig. 8. Derivatization was complete with

Table 6
Reproducibility of recovery from five different lots of human plasma

Plasma No.	Spiked concentration (ng ml^{-1})	Observed concentration (ng ml^{-1}) [a]			
		(+)-DU-124884	(−)-DU-124884	(+)-KC-9048	(−)-KC-9048
A	10.0	10.7 (+7)	10.7 (+7)	10.5 (+5)	10.5 (+5)
B	10.0	9.6 (−4)	9.6 (−4)	10.4 (+4)	10.3 (+3)
C	10.0	10.4 (+4)	10.3 (+3)	10.5 (+5)	10.5 (+5)
D	10.0	9.0 (−10)	9.0 (−10)	10.5 (+5)	10.6 (+6)
E	10.0	10.6 (+6)	10.6 (+6)	10.6 (+6)	10.5 (+5)
Mean		10.1 (+1)	10.1 (+1)	10.5 (+5)	10.5 (+5)
RSD (%)		7.5	7.4	0.9	0.9

[a] Relative errors (%) are indicated in parentheses.

25 μl of 1% (m/v) S-NEIC in chloroform. The derivatization reaction was immediate. A longer incubation time, investigated up to 60 min, did not have any adverse effect on the reaction. No underivatized (±)-DU-124884 and (±)-KC-9048 was observed using the direct HPLC on the chiral α-AGP column described above.

After derivatization, the solvent was evaporated under nitrogen and the residue suspended in 0.5 ml of water. Extraction of the formed diastereomers into diethyl ether is necessary in order to remove the interferences. The recovery data for (±)-DU-124884 and (±)-KC-9048 extracted from plasma are presented in Table 2. Around 90% of the compounds was recovered through the whole extraction process. We found that it was necessary to use polypropylene rather than glass tubes for the extraction to avoid the adsorption of (±)-KC-9048 on the glass. The results of comparison are also presented in Table 2. Polypropylene tubes were used throughout the study. When handling 80 samples the extraction takes about 5–6 h for one analyst.

3.3. Validation strategy

A few excellent analytical method validation guidelines for bioanalytical studies have appeared in the literature [17–22]. However, detailed guidelines for bioanalytical analyses of stereoisomer drugs are not available. Although it is not the purpose here to discuss guidelines or instructions for the bioanalysis of chiral drugs, a few issues on validation are addressed with emphasis on the establishment of stereospecificity of the method.

During the drug development stage, a large quantity of pure enantiomers may not be as easily available as the racemic compound. Usually, the purity of the racemate is higher and the potency of the racemate is better defined than that of the single enantiomers. For an LC method, if the separation of enantiomers is sufficient to allow the quantitation of small amounts of one enantiomer in the presence of its isomer at a disproportionate concentration, it is of advantage to validate some important parameters of a method such as the LOQ, precision, accuracy, linearity and parallelism using standards and QCs prepared from the racemates. On the other hand, in order to investigate interconversion of the enantiomers, solutions of QCs must be prepared from single enantiomers for stability studies. Enantiomers of high enantiometric purity are desirable for these studies, but their potencies are less important. For (±)-DU-124884 and (±)-KC-9048 method validation, racemic reference standard materials were used for most of the validation parameters. Additional QCs were prepared from the single enantiomer for stability tests to demonstrate the lack of stereoconversion.

3.4. Limit of quantitation (LOQ) and limit of detection (LOD)

LOQ was determined by extracting enantiomers of DU-124884 and KC-9048 at a concentration of

Table 7
Precision and accuracy of (±)-DU-124884 and (±)-KC-9048 parallelism quality controls

Dilution factor:	Parallelism quality control concentration (ng ml^{-1})				
	258			278	
	×4	×2		×4	×2
(+)-DU-124884			(+)-KC-9048		
Mean	64.3	134	Mean	66.6	134
RSD (%)	4.1	5.7	RSD (%)	4.0	1.0
RE (%)	0	+4	RE (%)	−4	−3
n	6	5	*n*	6	5
(−)-DU-124884			(−)-KC-9048		
Mean	64.2	134	Mean	66.8	134
RSD (%)	4.3	5.6	RSD (%)	3.9	0.7
RE (%)	0	+4	RE (%)	−4	−3
n	6	5	*n*	6	5

0.1 ng ml^{-1} in five replicates. The results of back-calculated concentrations are shown in Table 3. The RSDs were <15% and the bias for each individual measurement was −20 to +20%. The mean bias of five measurements was 0% for each enantiomer. The LOD was 0.02 ng ml^{-1} (signal-to-noise ratio = 3).

3.5. Precision, accuracy and linearity

Five validation curves with duplicate calibration standards were run on five separate days. One set of calibration standards were run at the beginning of the curve and the other set at the end. In order to mimic the situation of clinical sample analysis, each standard curve run was at least over 24 h of total chromatography time. Table 4 shows the accuracy and precision data at each individual standard concentration. The standards show a linear range of 0.1–200 ng ml^{-1}. The correlation coefficients of these curves are always >0.995. The concentration of over 250 ng ml^{-1} for each enantiomer was beyond the upper limit of the fluorescence detector. Therefore, 200 ng ml^{-1} was chosen as the upper limit of the standard curve. Table 5 presents the inter-day and intra-day accuracy and precision of QCs. The accuracy and precision data show that this method is consistent and reliable with low values of REs and RSDs for standards and QCs over the entire concentration range.

Table 8
Storage stability (−20 °C) of samples of (+)-DU-124884 and (+)-KC-9048 in human plasma [a]

Compound	Parameter	Low concentration (ng ml^{-1})		Medium concentration (ng ml^{-1})		High concentration (ng ml^{-1})	
		(+)	(−)	(+)	(−)	(+)	(−)
DU-124884	Initial	0.22	<0.02	4.5	0.06	122	2.2
		(6.0)		(2.5)	(7.6)	(11)	(11)
	After 43 days	0.21	<0.02	4.4	0.04	120	1.3
		(6.4)		(0.8)	(10)	(8.0)	(9.2)
	As % of initial	95.5		97.8		98.2	
	Conversion (%) [b]	0		0		0	
KC-9048	Initial	0.22	<0.02	5.0	0.09	131	3.2
		(5.3)		(5.9)	(14)	(3.5)	(4.4)
	After 43 days	0.24	<0.02	5.1	0.07	137	3.2
		(1.2)		(2.5)	(27)	(5.9)	(6.9)
	As % of initial	109.1		102.8		104.5	
	Conversion (%) [b]	0		0		0	

[a] Number of determinations = 3. RSDs (%) are given in parentheses.
[b] Conversion (%) = {[(−)f]/[(+)f] − [(−)i]/[(+)i]} × 100, where [(−)f] and [(−)i] are the final and initial concentrations of (−)-enantiomers, respectively, and [(+)f] and [(+)i] are the final and initial concentrations of (+)-enantiomers, respectively.

3.6. Selectivity

To achieve good selectivity, a method should lack interferences and matrix effect. Excellent reproducibility in recovery of the spiked compounds, reflected by the precision and accuracy, was obtained from all five different lots of plasma tested, indicating the lack of matrix effect. The results are shown in Table 6. All five lots of human plasma tested showed the absence of interferences.

3.7. Parallelism

Clinical samples may contain drug concentrations higher than the upper limit of the standard concentration range. These samples will be diluted with a blank matrix prior to analysis. The blank matrix lot used to dilute the clinical samples may not be the same lot as the clinical samples. Owing to physical–chemical interactions between the analytes and the compounds in the matrices, bias on the concentration determination after dilution may occur. A parallelism study was performed to ensure the lack of such matrix effects. Table 7 presents the precision and accuracy of parallelism QCs which were diluted two- and fourfold with control plasma from a different pool prior to use. Only −4 to +4% of bias was observed.

3.8. Stability

The protocol of the stability study should be designed to cover the conditions that the clinical samples may experience. For enantiomers, stability means the lack of conversion of the isomers and the lack of non-stereospecific or stereospecific degradation. Stabilities of sample storage, processing (freeze–thaw, benchtop) and chromatography (on-system and re-injection) were tested and established. Test (+)QCs spiked only with (+)-enantiomers were used for the stereoconversion test. Since the exact potencies other than enantiomeric purities, which were >98%, were not defined, the theoretical concentrations for these test (+)QCs were not calculated. This was not an issue because the purpose of this test was to determine whether the content of (+)-enantiomers has been changed and whether the (−)-enantiomers have formed upon storage, processing and chromatography. The observed concentrations were calculated using the calibration standards on the condition that the normal QCs on the same curve pass the acceptance criteria.

Sample storage stability was tested after (+)QCs had been stored in a freezer at −20°C for 43 days. The results are given in Table 8. The values for the stored samples were 95.5–98.2% and 102.8–109.1% of the corresponding values in the original assay for (+)-DU-124884 and (+)-KC-9048, respectively. For the (+)QCs of medium concentrations, the calculated (−)-enantiomer concentrations were between the LOQ (0.1 ng ml^{-1}) and LOD (0.02 ng ml^{-1}). However, in order to estimate the stereoconversion of the enantiomers, concentrations of (−)-enantiomers above the LOD were included. Even at these low concentrations, the RSD values were still acceptable. No conversion from the (+)-enantiomers into (−)-enantiomers was observed. The absence of significant amount of the (−)-enantiomers also indicated that no conversion occurred during the extraction and derivatization process.

(+)QSs were subjected to various cycles of freezing and thawing and the samples were then analysed. Both (+)-DU-124884 and (+)-KC-9048 were stable after multiple cycles of freezing and thawing. After two freeze–thaw cycles, the values for (+)QCs were 95.3–100% of that for one cycle for (+)-DU-124884 and 99.3–109.1% for (+)-KC-9048. After three freeze–thaw cycles, the values for (+)QCs were 94.2–104.5% of that for one cycle for (+)-DU-124884 and 96.2–109.1% for (+)-KC-9048. No conversion from the (+)-enantiomers into the (−)-enantiomers were observed. Good freezing and thawing stability indicates that the clinical samples can be re-assayed if necessary.

Some (+)QC samples were stored on the bench of the chemist for 2 h. The benchtop stability after 2 h at room temperature was 95.2–100% for (+)-DU-124884 and 95.8–100% for (+)-KC-9048 compared with the corresponding initial values. No conversion from the (+)-enantiomers into (−)-enantiomers was noticed. Good benchtop

stability allows the chemist to process a large numbers of samples with no degradation of the compounds.

On-system and re-injection stabilities are an indication of stability of the sample in the autosampler during a run. On-system stability was determined by injecting the (+)QC samples at the beginning and end of a run. Re-injection stability was determined by re-injecting the (+)QC samples which had been injected at the beginning of the run and had been exposed to the air. Good re-injection stability ensures that the clinical samples can be re-analyzed by re-injection. The on-system stabilities in the autosampler after 34 h were 92.3–104.5% and 92.2–100% for (+)-DU-124884 and (+)-KC-9048, respectively, compared with the initial values. Samples re-injected after 38 h were 92.3–104.5% and 94.1–104.3% of the original corresponding values for (+)-DU-124884 and (+)-KC-9048, respectively. No conversion from the (+)-enantiomers into (−)-enantiomers was noticed.

4. Conclusion

A rugged and sensitive LC method for the determination of DU-124884 and KC-9048 enantiomers in human plasma was developed. The unique extraction and derivatization process provides consistent and high recoveries for each enantiomer. Derivatization of the analytes with S-NEIC was immediate and complete. The formed diastereomers were separated on a silica column in the normal-phase mode and detected by fluorescence. As low as 0.1 ng ml^{-1} of each enantiomer in plasma can be quantified using 0.5 ml of plasma.

This LC method was validated to meet the pharmaceutical industry guidelines with additional tests on enantiomer conversion through storage, processing and chromatography. Linearity, precision and accuracy were determined. Consistent recoveries, parallelism and stabilities of storage, freeze–thaw cycles, benchtop, on-system and re-injection were established.

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