Development of High Performance Liquid Chromatographic Method with Pre-Column Derivatization for Determination of Enantiomeric Purity of Hydantoin-5-acetic Acid

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Summary: The objective of the present work was to develop and validate a rapid analytical method for determination of enantiomeric purity of hydantoin-5-acetic acid. The method was based on pre-column derivatization of hydantoin-5-acetic acid with phenylamine and complete separation of enantiomers has been achieved on a CHI-DMB analytical column (250×4.6 mm) using *n*-hexane: isopropanol (80:20 *v/v*) as mobile phase at a flow rate of 1.0 ml min^{-1} under UV and optical rotation (OR) detection. Detection wavelength was set at 254 nm. The effects of isopropanol, ethanol, column temperature and flow rate of the mobile phase on enantioselectivity and resolution of enantiomers were evaluated. The method was validated with respect to precision, accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness and stability. The present method is expected to be accurate, stable, rapid and sensitive and can be used for the determination of the enantiomeric purity of hydantoin-5-acetic acid in bulk samples.

Keywords: HPLC; enantiomeric purity; enantioseparation; hydantoin-5-acetic acid; pre-column derivatization; method validation.

Introduction

Hydantoin-5-acetic acid [2-(2,5-dioxoimidazolidin-4-yl)acetic acid] (Fig. 1) has one pair of enantiomers for an asymmetric center on its ring. It contains one rigid acceptor-donor-acceptor hydrogen-bonding site and a flexible carboxylic acid side chain, which can adopt different conformations. Since carboxylic acids form robust and directed hydrogen bonds [1], and also can form a most-studied synthon together with an amide [2], hydantoin-5-acetic acid and its derivatives have been used to search for new biologically active compounds. For example, Sham *et al*. [3] used it for synthesis of various amide derivatives, which exhibited good anti-inflammatory activity. James *et al*. [4] synthesized a series of novel hydantoins as structural alternatives to hydroxamate inhibitors of TACE. In 2009, Danylo *et al*. [5] tested the *in vitro* anticancer activity of its derivatives in National Cancer Institute.

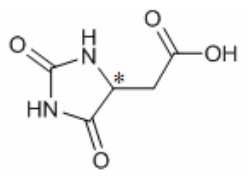

Fig. 1: Chemical structure of hydantoin-5-acetic acid.

The increasing demand for enantiopure drugs has led to the need for the preparation of new enantiomerically pure compounds. So it is very meaningful to determine the enantiomeric purity of hydantoin-5-acetic acid in pharmaceutical preparations. However, there was no resolution report for the enantiomers of hydantoin-5-acetic acid. Thus an efficient and economic method is necessary to be developed for precisely determination of enantiomeric excess (e.e.%) of the hydantoin-5-acetic acid enantiomers. Although there are many methods used for the quantitative analysis of drugs, liquid chromatography is a simple, sensitive and accurate method. Among the available HPLC methods for the separation of enantiomers, the application of chiral stationary phases (CSPs) has been known to be the most accurate, convenient, and economic means for the exact determination of the enantiomeric purity of chiral compounds [6-9]. In the method development, the selection of an appropriate CSP column is probably the most substantial challenge. With the use of certain columns, there are many problems in this connection include the instability of the CSP under some mobile phase conditions, restricted temperature ranges, pressure and flow-rate. Consequently, the availability of durable, stable and high-performance column is preferable in developing a rugged and reproducible method. Experimental results indicated that hydantoin-5-acetic acid can not be separated directly on the popular chiral columns satisfactorily. So in this paper, hydantoin-5-acetic acid was pretreated with derivatization reagents, and then the obtained derivatives were separated with chiral columns. The effects of some parameters, including organic modifier type and concentration, column temperature and flow rate, on the retention and

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enantioseparation were investigated.

Results and Discussion

Derivatization Conditions

In chiral liquid chromatography, enantiospecific interactions between a chiral selector and the enantiomers of an analyte resulting in transient diastereomeric complexes were required in order to achieve enantioseparations. The formation of the complexes was driven by dipole–dipole interactions, H-bonds, van der Waals interactions and π–π interactions. Considering that there was no strong ultraviolet absorption and π–π interaction group in the molecule of hydantoin-5-acetic acid, aromatic amines were used as derivatization reagents in order to detect with UV detector conveniently. At the same time, larger molecule could be obtained after derivatization, and the steric effect could enhance adsorption with CSP to improve chiral recognition. To study the π–π interactions between a chiral selector and the enantiomers of an analyte, a π–acidic or π–basic group would be contained in the molecules of the derivatives of hydantoin-5-acetic acid, so the aromatic amines such as phenylamine, 4-methylaniline, 3,5-dimethylaniline and 3-nitroaniline were chosen as derivatization reagents. For the instability of hydantoin-5-acetic acid, it is necessary to store it in a cool dry place. So it would be better to control the experimental temperature below 50°C in the whole process, to avoid the decomposition of the desired products.

Comparison of the Chiral Stationary Phases

The four derivatives of hydantoin-5-acetic acid were used in the method development, and four different chiral stationary phases were employed as follows: Chiralcel OD-H, CHI-DMB, Whelk-O1 and DNB-PG. The results indicated that the four derivatives of hydantoin-5-acetic acid could be separated on the CHI-DMB and Chiralcel OD-H columns (Table-1). It was found that the retention time of the four derivatives of hydantoin-5-acetic acid on the CHI-DMB column showed a more significant difference as compared with that of Chiralcel OD-H column. However, the average retention time of the four derivatives of hydantoin-5-acetic acid on the CHI-DMB column was shorter than that on Chiralcel OD-H column. The type of organic modifier was found to dramatically influence the retention and resolution of the four derivatives of hydantoin-5-acetic acid on both columns. After the replacement of isopropanol with ethanol, the peak shape became more sharply, but the selectivity and resolution of the enantiomers on the CHI-DMB and Chiralcel OD-H column did not improve apart from exceptional cases. Over all, the performance of the CHI-DMB column was superior to that of the Chiralcel OD-H column, so it was selected in further experiments.

Effect of Organic Modifier

As shown in Table-1, the retention and resolution of the phenylamine, 4-methylaniline and 3,5-dimethylaniline derivatives of hydantoin-5-acetic acid on the CHI-DMB column were approximate agreement, and the phenylamine derivative of hydantoin-5-acetic acid would be chosen as a typical sample. The resolution of the 3-nitroaniline derivative of hydantoin-5-acetic acid on the CHI-DMB column was the best, but the retention time was too long. So, the separation of the phenylamine and 3-nitroaniline derivatives of hydantoin-5-acetic acid on the CHI-DMB column should be further investigated.

Table-1: Resolution of racemic hydantoin-5-acetic acid derivatives on the CHI-DMB and Chiralcel OD-H columns.

Chiral column	Compound	*n*-hexane: isopropanol				*n*-hexane: ethanol			
		t_1	t_2	α	R_s	t_1	t_2	α	R_s
Kromasil CHI-DMB	1	24.811	46.801	2.035	4.024	11.541	18.171	1.832	1.973
	2	9.643	15.147	1.907	1.976	6.389	8.224	1.652	1.070
	3	8.939	13.589	1.867	1.890	6.133	7.712	1.617	0.934
	4	8.459	13.419	2.015	1.968	5.867	7.552	1.735	1.066
Chiralcel OD-H	1	24.245	27.584	1.162	0.421	17.984	19.147	1.081	0.221
	2	18.603	22.720	1.275	0.678	12.117	14.464	1.276	0.718
	3	22.699	22.699	1	0	13.344	14.059	1.073	0.182
	4	13.984	16.629	1.255	0.521	10.197	11.392	1.182	0.463

1: the 3-nitroaniline derivative of hydantoin-5-acetic acid, 2: the phenylamine derivative of hydantoin-5-acetic acid, 3: the 4-methylaniline derivative of hydantoin-5-acetic acid, 4: the 3,5-dimethylaniline derivative of hydantoin-5-acetic acid; mobile phase composition on the CHI-DMB column: *n*-hexane 80%; mobile phase composition on the Chiralcel OD-H column: *n*-hexane 85%; t_1: retention time of the first enantiomer; t_2: retention time of the second enantiomer; α: separation factor; R_s: resolution; flow rate: 1.0 ml min^{-1}; column temperature: 25°C; UV detection wavelength: 254 nm.

The type and concentration of organic modifier were found to influence the retention and resolution of the phenylamine and 3-nitroaniline derivatives of hydantoin-5-acetic acid on the CHI-DMB column dramatically. The selectivity and resolution of the enantiomers on the CHI-DMB column were investigated when isopropanol and ethanol were used as modifiers, respectively (shown in Table-2). Both organic modifiers showed good selectivity for the enantiomers. However, the retention times in ethanol were shorter than that in isopropanol. On decreasing the concentration of organic modifier, the capacity factors as well as resolutions were increased. As a compromise between resolution and retention time, 20% isopropanol in *n*-hexane was found to be an optimum mobile phase for the phenylamine derivative of hydantoin-5-acetic acid, and 40% isopropanol in *n*-hexane was found to be an optimum mobile phase for the 3-nitroaniline derivative of hydantoin-5-acetic acid. In the optimum mobile phase, the retention and resolution of the phenylamine and 3-nitroaniline derivatives of hydantoin-5-acetic acid were similar, and the phenylamine derivative of hydantoin-5-acetic acid was used for further studies. The chromatograms of the phenylamine derivative of hydantoin-5-acetic acid were shown in Fig. 2 and 3. It was found that (+)-phenylamine derivative of hydantoin-5-acetic acid enantiomer was eluted first.

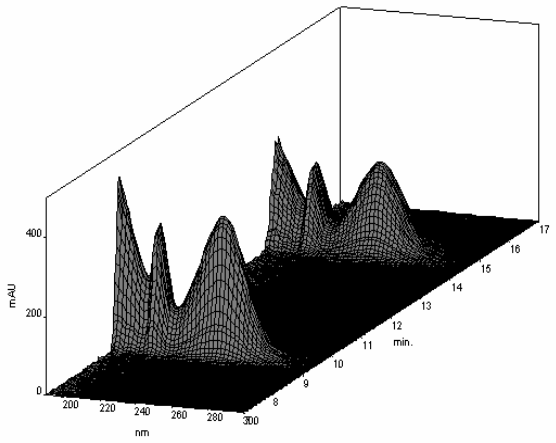

Fig. 2: Chromatogram of the phenylamine derivative of hydantoin-5-acetic acid enantiomers detected by photodiode-array detector. Conditions: stationary phase, CHI-DMB; mobile phase, *n*-hexane: isopropanol (80:20, *v/v*); flow rate, 1.0 ml min^{-1}; column temperature, 25 °C; UV detection wavelength, 254 nm.

Table-2: Effect of mobile phase composition on selectivity and resolution of the 3-nitroaniline and phenylamine derivatives of hydantoin-5-acetic acid enantiomers.

Mobile phase	Compound 1		Compound 2	
	α	R_s	α	R_s
***n*-hexane: isopropanol 85:15**	-	-	**1.853**	**2.293**
***n*-hexane: isopropanol 80:20**	**2.035**	**4.024**	**1.907**	**1.976**
***n*-hexane: isopropanol 75:25**	**2.037**	**2.979**	**1.938**	**1.614**
***n*-hexane: isopropanol 70:30**	**2.042**	**2.379**	**2.047**	**1.388**
***n*-hexane: isopropanol 65:35**	**2.068**	**2.066**	**2.139**	**1.211**
***n*-hexane: isopropanol 60:40**	**2.097**	**1.947**	**2.260**	**1.155**
***n*-hexane: isopropanol 55:45**	**2.118**	**1.690**	**2.463**	**1.036**
***n*-hexane: ethanol 90:10**	-	-	**1.616**	**1.932**
***n*-hexane: ethanol 85:15**	**1.825**	**2.707**	**1.622**	**1.474**
***n*-hexane: ethanol 80:20**	**1.832**	**1.973**	**1.652**	**1.070**
***n*-hexane: ethanol 75:25**	**1.855**	**1.574**	**1.763**	**0.949**
***n*-hexane: ethanol 70:30**	**1.911**	**1.345**	**1.951**	**0.822**

1: the 3-nitroaniline derivative of hydantoin-5-acetic acid, 2: the phenylamine derivative of hydantoin-5-acetic acid; α: separation factor; R_s: resolution; stationary phase: CHI-DMB; flow rate: 1.0 ml min^{-1}; column temperature: 25 °C; UV detection wavelength: 254 nm.

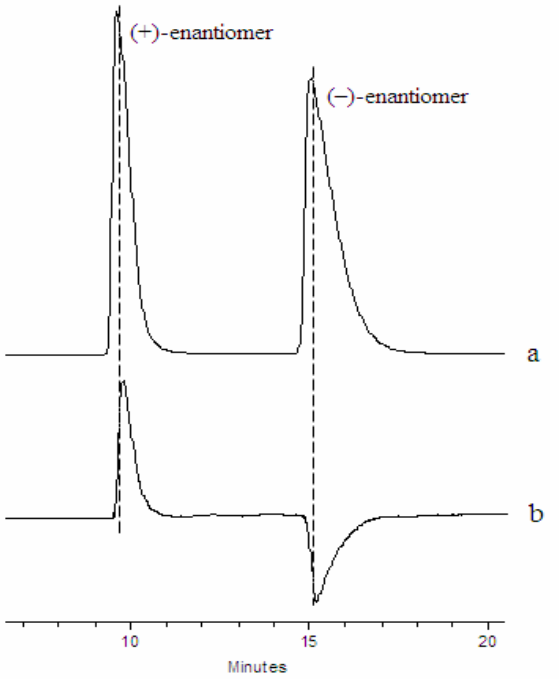

Fig. 3: Chromatograms obtained from the phenylamine derivative of hydantoin-5-acetic acid enantiomers on CHI-DMB. Conditions: mobile phase, *n*-hexane: isopropanol (80:20, *v/v*); flow rate, 1.0 ml min^{-1}; column temperature, 25 °C; detection wavelength, 254 nm.
a. UV detection
b. optical rotation detection

Effect of Temperature

In order to study the chiral recognition mechanism further, the effects of column temperature on selectivity and resolution of the phenylamine

derivative of hydantoin-5-acetic acid enantiomers were investigated. Temperature is an important factor to affect enantiomeric recognition processes [10, 11]. In the experiment, with the increasing of temperature, the retention time decreased. The logical explanation may be that the analytes on molecular level have lower adsorption as temperature increased and therefore migrate rapidly through the column [12]. According to the Van′t Hoff equation [12-15]:

$$\ln k = -\frac{\Delta H^{\theta}}{RT} + \frac{\Delta S^{\theta}}{R} \qquad (1)$$

where k is the retention factor, R is the gas constant and T is the absolute temperature in Kelvin, ΔH^{θ} and ΔS^{θ} are the molar enthalpy and molar entropy of adsorption. Van't Hoff plots were drawn for logarithm of retention factor (ln k) versus inverted temperature ($1/T$) for the two enantiomers, which yielded straight lines in the temperature range 288~323K (15~50°C). (Fig. 4) ΔH^{θ} and ΔS^{θ} for the two enantiomers were obtained from the slope and intercept of the straight lines, respectively. The change in free energy ($\Delta\Delta G^{\theta}$) accompanying the separation of two enantiomers was given by

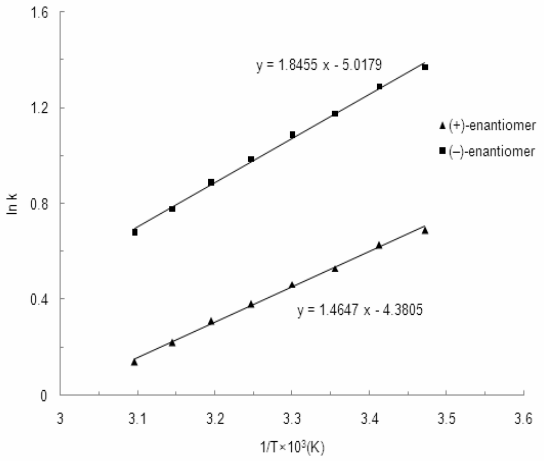

Fig. 4: Plot of ln k versus $1/T$. Conditions: Mobile phase, *n*-hexane: isopropanol (80:20, *v/v*); flow rate, 1.0 ml min^{-1}; UV detection wavelength, 254 nm.

$$\Delta\Delta G^{\theta} = \Delta\Delta H^{\theta} - T\Delta\Delta S^{\theta} \qquad (2)$$

The apparent thermodynamic parameters for the phenylamine derivative of hydantoin-5-acetic acid enantioseparations were obtained with *n*-hexane: isopropanol (80:20 *v/v*) as the mobile phase. The corresponding data were listed in Table-3, which proved that the processes of enantiomeric recognition were enthalpy-controlled in the study.

Table-3: Thermodynamic data calculated from the Van′t Hoff plots of the phenylamine derivative of hydantoin-5-acetic acid enantiomers in temperature rang 288~323K (15~50°C).

Enantiomer	ΔH^{θ} (kJ mol^{-1})	$\Delta\Delta H^{\theta}$ (kJ mol^{-1})	ΔS^{θ} (J K^{-1} mol^{-1})	$\Delta\Delta S^{\theta}$ (J K^{-1} mol^{-1})	$\Delta\Delta G^{\theta}$ (kJ mol^{-1})
(+)-enantiomer	-12.178	-3.166	-36.420	-5.299	-1.586 (298K)
(−)-enantiomer	-15.343		-41.719		

Effect of Flow Rate

The effect of flow rate on resolution of the phenylamine derivative of hydantoin-5-acetic acid enantiomers was investigated in the range of 0.5~1.5 ml min^{-1} (mobile phase was *n*-hexane: isopropanol (80:20 *v/v*) and column temperature was 25°C). The results were shown in Table-4, and it can be found that with the increase of the flow rate, the separation factor increased but the resolution decreased. On the comprehensive consideration of separation factor and resolution, 1.0 ml min^{-1} was chosen as the optimal flow rate.

Table-4: Effect of flow rates on enantioselectivity.

Flow rate (ml min^{-1})	k_1	k_2	α	R_s
0.5	4.314	7.353	1.704	2.376
0.8	2.329	4.227	1.815	2.004
1.0	1.699	3.239	1.907	1.976
1.2	1.215	2.478	2.039	1.924
1.5	0.767	1.771	2.307	1.748

k_1: capacity factor of the first enantiomer; k_2: capacity factor of the second enantiomer; α: separation factor; R_s: resolution; stationary phase: CHI-DMB; column temperature: 25°C; mobile phase: *n*-hexane: isopropanol (80:20, *v/v*); UV detection wavelength: 254 nm.

Chiral Recognition Mechanism

As shown in Table-1, the resolution of the 3-nitroaniline derivative of hydantoin-5-acetic acid was 4.024, which was the best. The phenylamine, 4-methylaniline and 3,5-dimethylaniline derivatives of hydantoin-5-acetic acid were resolved worse. These results clearly demonstrated the importance of the effective π–π donor–acceptor interaction between the π–basic 3,5-dimethylbenzoyl group of the chiral selector on CHI-DMB column and the π–acidic 3-nitrophenyl group of analyte for the chiral recognition. And the contribution of H-bond interaction between the chiral selector and the analyte to the chiral recognition was also very important. As

a result, a simple chiral recognition mechanism model focusing on the importance of the π–π donor–acceptor interaction and the H-bond interaction between the chiral selector on CHI-DMB column and the analyte to the chiral recognition was proposed, as shown in Fig. 5.

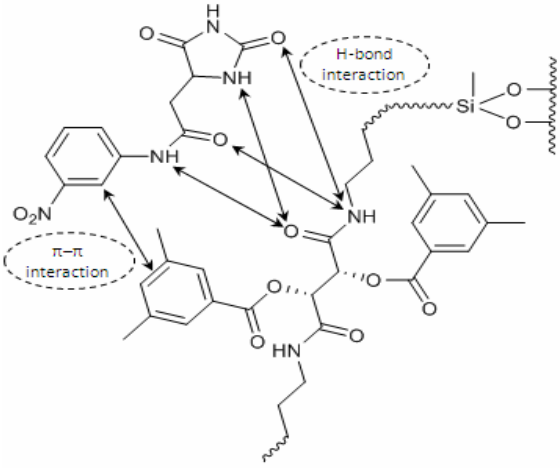

Fig. 5: The interaction sites between the derivatives of hydantoin-5-acetic acid and the chiral selector on CHI-DMB column.

Validation of HPLC Method

The HPLC method was validated with regard to precision, accuracy, linearity, limit of detection (LOD) and limit of quantitation (LOQ), robustness and stability. In the validation of HPLC method, the sample solutions from hydantoin-5-acetic acid were prepared and analyzed by using the optimized derivatization procedure and chromatographic conditions.

Precision

Precision of the method was tested with preparing five individual solutions of the phenylamine derivative of hydantoin-5-acetic acid and making triplicate injections for each solution under the working conditions. The RSD% of the assay was less than 1.23%. Inter and intra-day assay precisions were performed with analyzing the solutions for five times in a day for 3 days. The RSD% of the assay was less than 1.38% for both the isomers.

Accuracy

Accuracy studies were performed with spiking the phenylamine derivative of hydantoin-5-acetic acid solution at six levels with respect to specified level and analyzing each solution in triplicate (n=3) for 3 days. The recoveries were between 99.2% and 102.1% with percentage relative standard deviation less than 1.27%.

Linearity

For the determination of linearity, standard calibration curve was used, which was linear over the concentration range of 30~1000 μg ml^{-1}. The calibration curves shown good linearity with r_1^2 = 0.9998 and r_2^2 = 0.9996 and the regression equations for the first enantiomer and the second enantiomer were y_1 = 13820.4 x_1 - 7501.5 and y_2 = 14174.6 x_2 - 6243.9, respectively.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were estimated at signal-to-noise ratios of 3:1 and 10:1, respectively by injecting a series of dilute solutions. LOD was found to be 3.9 and 4.9 μg ml^{-1} for the first enantiomer and the second enantiomer, respectively. LOQ was found to be 8.1 and 9.9 μg ml^{-1} for the first enantiomer and the second enantiomer, respectively.

Robustness

Robustness of the method was studied with making small deliberate changes in the method parameters. A variation of 2% of isopropanol in the composition of the mobile phase hardly affected the resolution except that retentions were changed. The effect of temperature was studied with analyzing sample at 25±2°C. Again retention times varied in the range of 1 min but the resolution remained above 1.9. The effect of flow rate was studied with analyzing the samples in 0.8 and 1.2 ml min^{-1} flow rates. In the both cases the resolution was found to be above 1.9.

Stability Studies

In the experiment, the RSD of the phenylamine derivative of hydantoin-5-acetic acid content at room temperature was 1.13%. When the sample solution was heated at 50°C, no decomposition product was observed in the chromatogram. However, no peak corresponding to the phenylamine derivative of hydantoin-5-acetic acid was detected which suggested it was hydrolyzed when treated with acid and base.

Experimental

Chemicals and Reagents

(Rac)-hydantoin-5-acetic acid (98% of chemical purity) was purchased from Alfa Aesar (Tianjin, China). *n*-Hexane, ethanol and isopropanol of HPLC grade were supplied by Hangjia Chemical Reagent Co., Ltd. (Chengdu, China). Phenylamine, 4-methylaniline, 3,5-dimethylaniline, 3-nitroaniline and isobutyl chloroformate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Chengdu, China). Triethylamine was purchased from Kelong Chemical Reagent Co., Ltd. (Chengdu, China). Other reagents supplied by Hangjia Chemical Reagent Co., Ltd. (Chengdu, China) were all in analytical level.

Instruments and Equipments

Analysis was carried out on a Shimadzu series liquid chromatography system, equipped with LC-20AT pump, SPD-M20A photodiode array detector (Kyoto, Japan), JASCO (Japan) model OR-2090 optical rotation detector, and an HCT-360 LC column box (Tianjin, China). Chromatographic parameters such as peak areas, retention times, theoretical plates, *etc*. were calculated using the Class-VP workstation.

Chromatographic Conditions

Chiralcel OD-H (250×4.6 mm; particle size 5 μm) (Daicel, Japan), DNB-PG (250×4.6 mm; particle size 5 μm), Whelk-O1 (250×4.6 mm; particle size 5 μm) (Regis Technologies, USA) and Kromasil CHI-DMB (250×4.6 mm; particle size 5 μm) (Akzo Nobel, Sweden) were used for the separation. Mobile phase consisted of *n*-hexane: isopropanol or ethanol and the column temperature was 25°C. The flow rate was 1.0 ml min^{-1} and the detection wavelength was kept at 254 nm. Void times were determined using ethanol as a marker. The injection volume was about 10 μl. The sample solution was prepared by dissolving the sample in isopropanol at 200 μg ml^{-1}.

Derivatization Procedure

The four derivatives of hydantoin-5-acetic acid were obtained according to the general procedure in Fig. 6. A solution of (Rac)-hydantoin-5-acetic acid (158.1 mg, 1 mmol) in THF (30 mL) was stirred at 0°C, to which triethylamine (1.2 equiv) was added. After 3 minutes, isobutyl chloroformate (1.2 equiv.) was added dropwise to the solution, and the mixture was stirred for 10 minutes. Next, amine (1.1 equiv) was added to the solution, and the mixture was stirred for another 30 minutes. Subsequently, the mixture was allowed to warm to room temperature overnight. After the completion of the reaction, the THF was removed under reduced pressure. The residue left behind was dissolved in CH_2Cl_2 (30 mL) and washed thoroughly with 1M HCl, saturated $NaHCO_3$ solution, and brine, dried over sodium sulfate, and concentrated. Further purification of the desired product was accomplished by column chromatography on silica gel, eluting with ethyl acetate/petroleum ether (2:1, v/v), to obtain white powders as LC analytical sample.

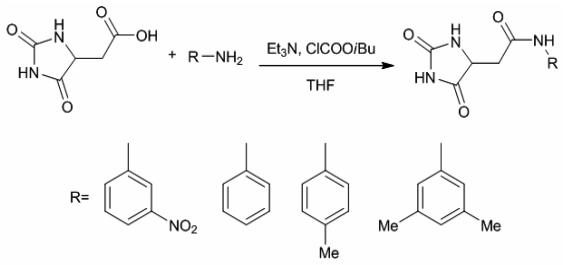

Fig. 6: The derivatization reaction route of hydantoin-5-acetic acid.

Stability Studies

Stabilities of the derivatives of hydantoin-5-acetic acid were investigated under various conditions as follows. For the reaction under room temperature, the sample solution in a tightly capped volumetric flask was put on a laboratory bench at room temperature for two days. The derivatives of hydantoin-5-acetic acid contents were checked at six-hourly intervals during this period. For the thermolysis, the solution was heated at 50°C for 1 h. For the acidic and basic hydrolysis, 10 mg the derivatives of hydantoin-5-acetic acid were dissolved in 5 ml isopropanol and heated at 50°C for 1 h after adding: (a) 5 ml 0.1 M HCl for acidic hydrolysis, (b) 5 ml 0.1 M NaOH for basic hydrolysis.

Conclusion

A method for enantiomeric separation and purity determination of hydantoin-5-acetic acid was developed on HPLC after pre-column derivatization. Under optimum derivatization reaction and chromatographic conditions, the enantiomers were well separated on CHI-DMB column. The effects of mobile phase composition and temperature on the resolution and retention of enantiomers have been

investigated to optimize the HPLC conditions. The enantiomeric separation was found to be an enthalpy driven process. The method was completely validated and shown satisfactory results for all the method validation parameters (precision, accuracy, linearity, LOD, LOQ, robustness and stability studies) tested. The method was proved to be useful for quantitative analysis of enantiomeric purity of the hydantoin-5-acetic acid in bulk samples.

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