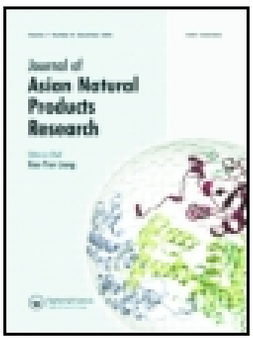

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A new pre-column derivatization for valienamine and beta-valienamine using o-phthalaldehyde to determine the epimeric purity by HPLC and application of this method to monitor enzymatic catalyzed synthesis of beta-valienamine

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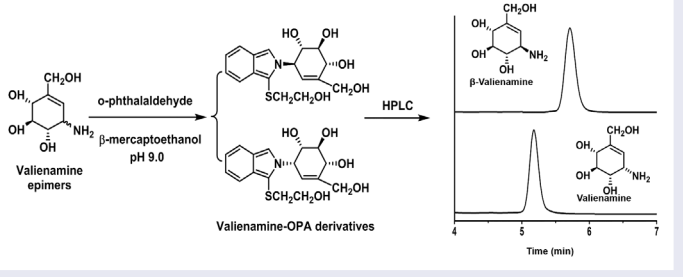
ABSTRACT

Valienamine and β-valienamine are representative C_7 N aminocyclitols with significant glycosidase inhibition activity that have been developed as important precursors of drugs for diabetes and lysosomal storage diseases, respectively. The quantitative analysis of these chiral compounds is crucial for asymmetric *in vitro* biosynthetic processes for converting valienone into valienamine epimers using aminotransferase. Here, we developed an efficient and sensitive method for separation and quantitative analysis of chiral valienamine using reversed-phase high-performance liquid chromatography (HPLC) through *o*-phthalaldehyde (OPA) pre-column derivatization of the analytes. The epimers were derivatized by OPA in borate buffer (pH 9.0) at room temperature for 30 s, separated on an Eclipse XDB-C18 (5 µm, 4.6 × 150 mm) column, eluted with 22% acetonitrile at 30 °C for 18 min, and detected by a fluorescence detector using 445 nm emission and 340 nm excitation wavelengths. The average resolution of the epimers is 3.86, and the concentration linearity is in the range of 0.02–20 µg/ml. The method proved to be effective, sensitive, and reliable with good intra- and inter-day precision and accuracy, and successfully evaluated the enantiopreference and catalytic capability of the potential aminotransferases on an unnatural prochiral substrate, facilitating the design of an asymmetric biosynthetic route for optically pure valienamine and β-valienamine.



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1. Introduction

Valienamine and β-valienamine (1-*epi*-valienamine) are both C_7 N aminocyclitols, which have pseudosugar structures similar to pyranoses, except for the ring oxygen being replaced with a carbon atom. They serve as optically building blocks for the synthesis of the inhibitors of glycosidases [1,2]. Valienamine (Figure 1(a)) exhibits α-glycosidase inhibition activity and has been determined to be the key moiety in the crop protectant validamycin A and the anti-diabetic drug acarbose [3,4]. As an important pharmaceutical precursor, valienamine can be chemically converted into valiolamine [5] and further modified to voglibose [6], which is another popular clinical therapeutic for type II diabetes [7]. β-Valienamine (Figure 1(b)) shows promising β-glycosidase inhibitor activity for the opposite amino group stereochemistry [8,9]. Its two derivatives, *N*-octyl-β-valienamine and *N*-octyl-4-*epi*-β-valienamine, have been proven to be promising chemical chaperone therapeutic agents for lysosomal storage diseases, including Gaucher disease, G_{M1}-gangliosidosis, and Morquio B disease caused by the disorder of β-glycosidase [10,11].

The chemical synthetic routes for valienamine and β-valienamine are quite challenging due to poor stereospecificity of chemical catalysts. The routes also suffer from multiple steps, harsh reaction conditions, and a proclivity to generate chemical pollutants [12,13]. An asymmetric biosynthetic strategy starting from the prochiral substrate valienone and by employing aminotransferase to produce optically pure valienamine or β-valienamine, based on the stereoselectivity of the candidate enzyme (Figure 2), was designed as a prospective alternative. Hence, a reliable separation and quantitative analysis method is required to separate the valienamine and β-valienamine epimers to evaluate the stereospecificity of the enzymatic products.

HPLC is a top choice for detection of chemicals with high precision and accuracy, and is superior to traditional thin-layer chromatography (TLC) analysis of valienamine [14]. A pre-column derivatization HPLC analysis method for valienamine was reported previously by Shen and co-workers [15]. Valienamine was detected and quantified through a pre-column derivatization procedure using *p*-nitrofluorobenzene and a UV detector, which required a harsh derivatization protocol using high temperatures (100 °C) for 30 min, with anhydrous samples redissolved in dimethylformamide. Kim's group described a quantitative analysis of valiolamine, a homologue of valienamine, derivatized with phenylisocyanate (PHI) [16]. That process also suffered from the disadvantage of a tedious procedure wherein the samples had to be dried and redissolved in dimethyl sulfoxide before analysis because

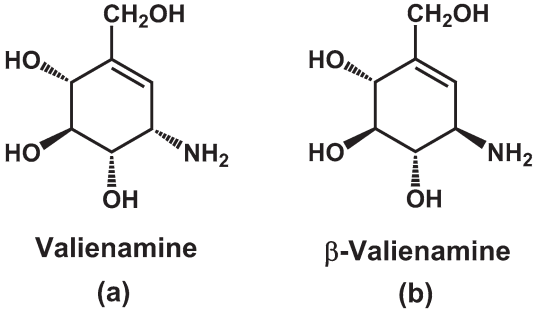

Figure 1. Structures of (a) valienamine and (b) β-valienamine.

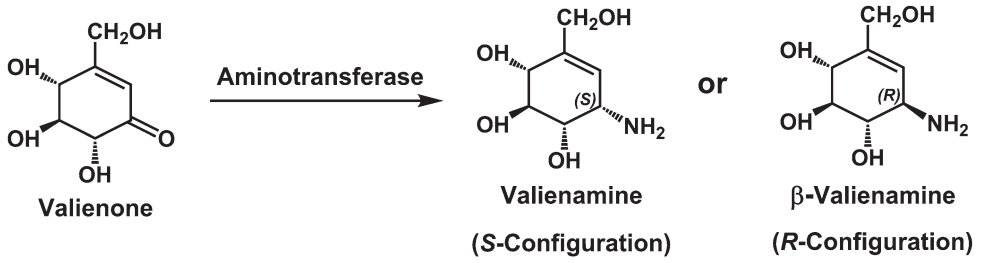

Figure 2. Asymmetric synthetic strategy for optically pure valienamine epimers by aminotransferase from the prochiral substrate.

PHI is sensitive to water. Most recently, Wang's group improved the method with 4-methoxybenzenesulfonyl fluoride (MOBS-F) as the derivatization reagent, which can react with valiolamine in aqueous solution [17]. However, it required a highly acidic solution as the elution mixture, consisting of 85% phosphate buffer (pH 3.0), which is especially damaging to the column and affects downstream mass spectrum signals. No other research on HPLC analysis for valienamine and β-valienamine epimers has been reported to date.

In this study, a reliable, sensitive, and facile HPLC analysis procedure is developed through pre-column derivatization by *o*-phthalaldehyde (OPA) and detection by a fluorescence detector (FLD). It is not only suitable to evaluate the asymmetric biosynthesis of chiral amines such as valienamine and β-valienamine, but can also conveniently connect in series with mass spectrometry (MS) for the identification of unknown chemicals and natural products with primary amino groups.

2. Results and discussion

2.1. Establishment of the derivatization conditions

Derivatization by chromophoric reagents is essential for the analysis of valienamine epimers because of their high polarity and because the absence of chromophores or fluorophores in their structures causes difficulty in both separation and detection processes. The derivatization was designed around the amino group of the valienamine epimers. OPA has been selected as the chiral derivatization reagent for amino acid analysis [18,19] over other derivatization reagents as its derivatization reaction is very facile, allowing an online process and thereby eliminating errors from manual operation and shortening the time interval between derivatization and detection. Furthermore, the derivatization reaction only occurs with primary amines, thereby eliminating the interference of secondary amines in complex samples such as microbial media. Considering these advantages, OPA was suitable for the separation and quantification of valienamine and β-valienamine by monitoring the formation of *R,S*-configuration chiral amino groups on the hexatomic ring of the prochiral substrate via the derivatization reaction shown in Figure 3.

To improve the efficiency of the derivatization for better detection sensitivity, the effects of the derivatization temperature, buffer pH, and reaction time were investigated by an $L_{25}(5^6)$ orthogonal experimental design with three factors and five levels at fixed concentrations of valienamine (10 μg/ml) and OPA (0.2 M). The reaction temperature was tested from 20 to

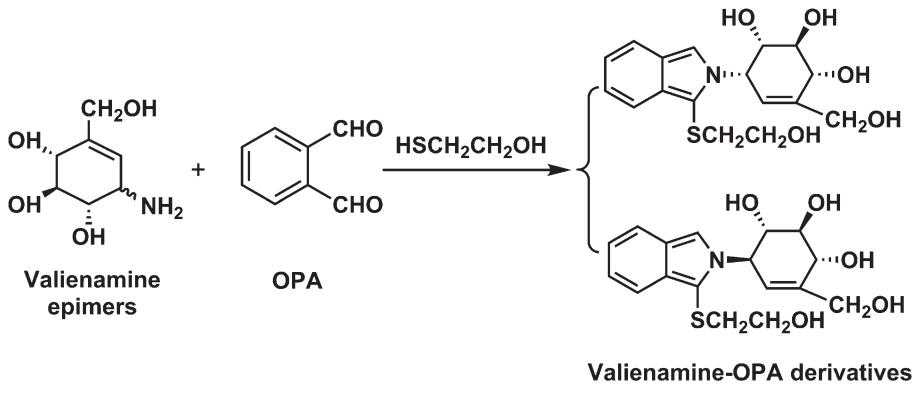

Figure 3. Scheme for the putative reaction of the derivatization of valienamine and β-valienamine with OPA.

40 °C, buffer pH from 8 to 12, and reaction time from 30 to 150 s by the gradients shown in Table 1.

Comparing the chromatographic peak area data, the results of the orthogonal test and range analysis showed the following order of the relative importance of the reaction factors: pH > temperature > time. Reaction pH was the most notable factor, and the optimal derivatization pH was 9.0. The reaction temperature and time had only a slight impact on the derivatization process.

Using the optimal conditions determined above, the concentration of OPA was tested from 0.01 to 0.20 M at room temperature for 30 s, and it was seen that the peak area of the valienamine derivative products reached a plateau at 0.1 M OPA (Figure 4). Taking into account all factors and the possible consumption of OPA by the amino donor L-glutamine in the enzymatic asymmetric synthetic system, as well as amino acids in the microbial culture medium, the optimal online derivatization conditions were 0.15 M OPA in a 0.40 M borate buffer (pH 9.0) at room temperature for 30 s before separation.

2.2. Establishment of the separation and detection conditions

To obtain the most sensitive detector response, the optimal wavelength for FLD was investigated. The optimal emission wavelength was determined to be 445 nm by scanning from 280 to 600 nm with the excitation wavelength fixed at 240 nm. Then, the excitation wavelength was determined to be 340 nm by scanning from 214 to 400 nm with the emission wavelength fixed at 450 nm. The characteristic emission wavelength was determined at 445 nm and excitation wavelength at 340 nm.

Unlike drug quality control, biosynthetic or fermentation process monitoring always needs to be combined with mass spectrometry for active component discovery and identification. Considering that a high concentration borate buffer is especially damaging to the column and also influences the MS signal, the mobile phase was prepared with (A) ultra-pure water and (B) 100% acetonitrile.

The two epimers were applied to the chromatographic system in pure form to obtain their individual elution profiles, and the effects of different experimental conditions on the resolution of the 1:1 epimer mixture were investigated. The resolution of the valienamine

Table 1. Factors of the orthogonal test for the derivatization of valienamine.

Level	Temp (°C)	pH	Time (s)
1	20	8	30
2	25	9	60
3	30	10	90
4	35	11	120
5	40	12	150

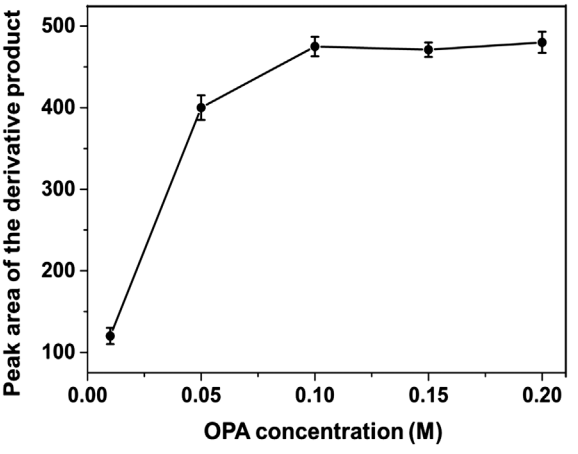

Figure 4. Effect of concentration of OPA on the amount of valienamine derivatives.

epimers was observed to be more sensitive to the mobile phase gradient ratio and flow rate than column temperature. Satisfactory separation was obtained with β-valienamine, which shows a longer retention time than valienamine under the chosen conditions.

Volume ratios of acetonitrile in the total mobile phase from 10 to 50% were examined, and the separation profiles were compared. The results showed that the retention time of the derivatives decreased with increasing acetonitrile concentration. Flow rates between 0.5 to 1.0 ml/min afforded appropriate separation resolution. Flows rate greater than 1.5 ml/min or lower than 0.4 ml/min led to overlapped peak forms. The optimal separation gradient parameters determined for the valienamine epimers – considering both resolution and efficiency of different experimental conditions – are given in Table 2, which shows that the retention times of valienamine and β-valienamine were 5.1 and 5.7 min with a resolution of 3.67 at an epimer concentration of 10 μg/ml (Figure 5(a)).

The same characteristic ion peaks, with *m/z* 352.1209 and 374.1027, were observed in the mass spectra of the two epimers under Electrospray Ionization (ESI) positive scan mode, corresponding to the two peaks of HPLC. These matched well with the theoretical values for $[M + H]^+$ and $[M + Na]^+$ ions of the valienamine and β-valienamine derivative products (Figure 5(b)). This evidence indicated that valienamine and β-valienamine were successfully derivatized by OPA and separated under optimal elution conditions. The entire procedure for epimer analysis was approximately 20 min, including derivatization, separation, and detection, which is more efficient than previous methods.

Table 2. HPLC gradients for the separation of the epimers.

	Time (min)			
Solvent (%)	0	8	12	18
Ultra-pure water I	78	0	78	78
Acetonitrile	22	100	22	22

Note: Flow rate: 1.0 ml/min, column temperature 30 °C.

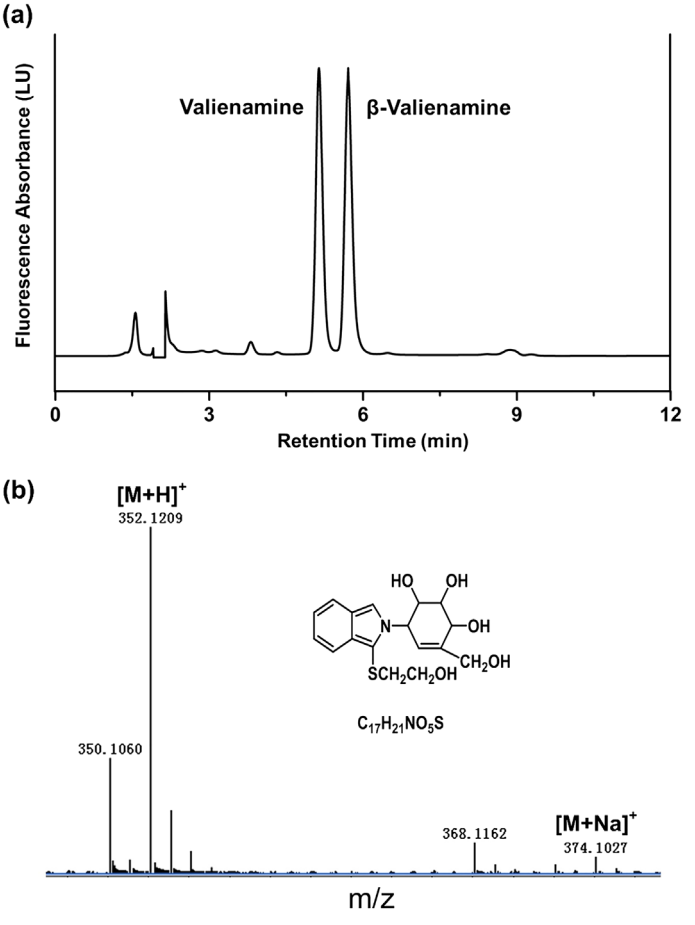

Figure 5. Separation and identification of valienamine epimer derivatives. (a) Separation of valienamine and β-valienamine at 10 μg/ml; (b) positive ESI mass spectra of the OPA-derivatized valienamine, which has the same characteristic ion peaks as the derivatized β-valienamine.

2.3. Validation and verification

2.3.1. System suitability parameters

Valienamine and β-valienamine showed average resolution of 3.86 ± 0.008 in 0.02–20 μg/ml of the 1:1 mixtures under the optimized conditions, with good peak shape and high column efficiency as expressed by the asymmetry factors and theoretical plate numbers shown in Table 3.

Table 3. Validation parameters of the method.

	Valienamine	β-Valienamine
Asymmetry factor (As)[a]	0.8285 ± 0.018	0.8395 ± 0.04
Resolution (R)[a]	3.86 ± 0.008	3.86 ± 0.008
Theoretical plate number (N)[a]	19,238	19,520
Linear range (μg/ml)	0.02–20	0.02–20
Regression equation	$y = 120.42x + 0.8726$	$y = 114.35x + 0.9533$
R^2	0.9994	0.9997
LOD (μg/ml)	0.002	0.002
LOQ (μg/ml)	0.01	0.01
Intra-day precision (% R.S.D., n = 6)[b]	1.22	1.46
Inter-day precision (% R.S.D., n=6)[b]	1.73	1.69
Standard recovery (%, n=9)	102.69	98.82
Spiked samples recovery (%, n=9)	100.43	101.09

[a]System suitability parameters were evaluated at epimer concentrations of 0.02–20 μg/ml.
[b]Intra-day and inter-day precision was calculated at an epimer concentration of 10 μg/ml.

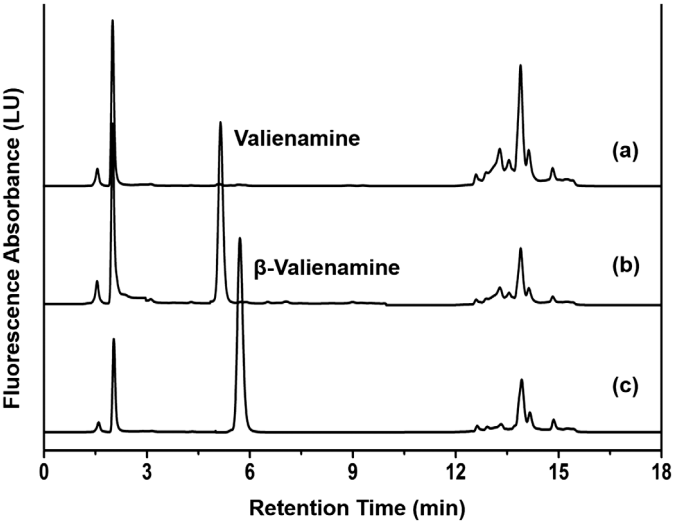

Figure 6. Chromatograms of OPA derivatives. (a) Blank reaction system; (b) blank reaction system spiked with valienamine, and (c) spiked with β-valienamine.

2.3.2. Selectivity

An asymmetric synthetic reaction system without any enzyme was separated and detected as a blank control. The chromatographic baseline from 3 to 12 min was stable (Figure 6(a)), and the spiked valienamine and β-valienamine were eluted at 5.1 (Figure 6(b)) and 5.7 min (Figure 6(c)), respectively. This indicated that the derivatives in the blank reaction system did not interfere with the separation and detection of the valienamine epimers.

2.3.3. Linearity and sensitivity

The linearity and sensitivity of the method were evaluated under the optimal derivatization and separation conditions, using gradual concentrations of the epimers as the horizontal coordinate (x) and the peak area as the vertical coordinate (y) to calculate the regression equation for the standard curves. The linearity test of the calibration range was carried out over the range of 0.02–20 μg/ml with the standard epimers using FLD. The calibration

curves were linear in the studied range with good correlation coefficients, and the linear regression equations for the corresponding curves are shown in Table 3.

The limit of detection (LOD) and limit of quantification (LOQ) were defined as S/N = 3 and 10, respectively. The LOD of the epimers detected by FLD was 0.002 μg/ml for the standard mixture solution. The LOQ was 0.01 μg/ml, which is a high sensitivity for FLD.

2.3.4. Precision and accuracy

Six replicate samples were averaged on the same day and over six continuous days to determine intra-day and inter-day precision values, respectively. The relative standard deviations (% R.S.D.) for the intra-day precision of valienamine and β-valienamine were 1.22 and 1.46, and for the inter-day precision, the values were 1.69 and 1.73, respectively.

The accuracy of the epimers was expressed in terms of absolute recoveries of standards and spiked samples in the blank enzymatic reaction system. Concentrations of 0.05, 1.0, and 8.0 μg/ml were detected by FLD. Every sample was repeated three times, and the average recoveries are shown in Table 3, reflecting the reproducibility and precision of the method.

2.4. Determination of epimers in the enzymatic reaction system

Biocatalysts have emerged as a powerful alternative to traditional chemical catalysts, especially for asymmetric synthesis [20,21]. One key requirement during process development is the discovery of a biocatalyst with appropriate enantiopreference and enantioselectivity [22,23]. We focused our efforts to develop a biosynthetic strategy for optically pure valienamine and β-valienamine through the prochiral substrate, which was deeply reliant on the precise analysis of the epimers.

Sixteen candidate sugar aminotransferases from different bacteria [24,25] were tested for their catalytic ability to produce chiral valienamine or β-valienamine. Mixtures of the enzymatic reaction samples were diluted and analyzed under the optimal conditions, and six aminotransferases from different sources were found to recognize the unnatural substrate valienone and showed different characteristic enantiopreferences (Table 4). Among these, BtrR (Figure 7(a)), Per, DesI, and WbpE demonstrated stringent stereospecificity with >99.9% epimeric purity to produce β-valienamine, but with different catalytic ability. Other candidates had lower stereospecificity and produced an epimer mixture. For example, ArnB (Figure 7(b)) from *Escherichia coli* gave a 60.1% epimeric purity for the β-valienamine product.

The analytical method developed herein allowed us to successfully evaluate the enantiopreference and catalytic capability of the candidate aminotransferases on the unnatural substrate while designing the asymmetric biosynthetic route for valienamine and β-valienamine.

Table 4. Catalytic properties of candidate enzymes used to produce β-valienamine.

Enzyme	Source	Valienamine (μg/ml)	β-Valienamine (μg/ml)	Epimeric purity (%)
BtrR	*Bacillus circulans*	–	1875.7	>99.9
Per	*Caulobacter crescentus*	–	191.6	>99.9
DesI	*Streptomyces venezuelae*	–	173.2	>99.9
WbpE	*Pseudomonas aeruginosa*	–	151.3	>99.9
NtdA	*Bacillus subtilis*	26.0	161.7	72.3
ArnB	*Escherichia coli*	42.8	172.0	60.1

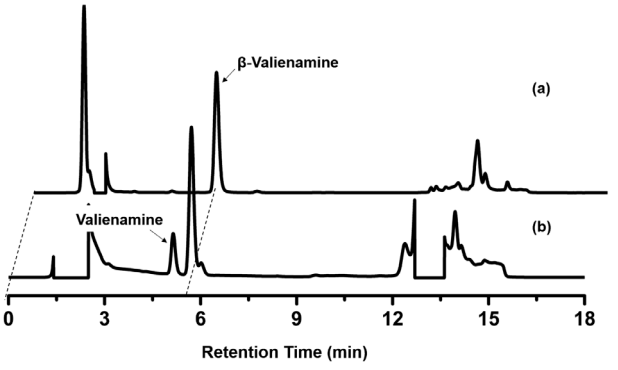

Figure 7. HPLC analysis of the enzymatic reaction under the optimal conditions. Different candidate enzymes produced (a) optically pure β-valienamine (BtrR) and (b) an epimer mixture (ArnB).

3. Experimental

3.1. Chemicals and reagents

Valienamine was purchased from Carbosynth China Ltd. (Suzhou, China). Valiolamine was from YaoXing Pharmaceutical Technology Co., Ltd. (Wuxi, China). β-valienamine was purified through an enzymatic reaction with the structure and purity (98%) verified by NMR. *o*-Phthalaldehyde (OPA) and β-mercaptoethanol (β-ME) were purchased from Sigma-Aldrich Shanghai Trading Co., Ltd. (Shanghai, China). Acetonitrile was from ANPEL Laboratory Technologies Inc. (Shanghai, China), and the other chemicals were purchased from Sigma-Aldrich (St Louis, USA). All chemicals were of analytical reagent grade, unless stated otherwise.

3.2. Standard solution preparation

The standard solution was prepared by dissolving valienamine and β-valienamine in distilled water to give a final concentration of 200 μg/ml. The solution was successively diluted with distilled water to create a concentration gradient from 0.2 to 200 μg/ml.

3.3. Sample preparation for the enzymatic reaction

The enzymatic asymmetric synthesis reaction system was composed of 5 mM valienone (prochiral substrate), 5 mM L-glutamine (amino donor), and 0.3 mM pyridoxal 5′-phosphate (cofactor). All the chemicals were incubated in 20 mM potassium phosphate (pH 7.5) with the purified candidate aminotransferase at 37 °C for 3 h. The reaction was terminated by methanol as a protein precipitation agent and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered through 0.22 μm membranes before analysis.

Spiked samples were prepared as control blanks using the protocol for making asymmetric synthetic reaction solutions, but without the addition of enzyme.

3.4. Chromatographic analysis

Chromatographic separation and detection was performed on an Agilent 1260 Infinity HPLC system with a quaternary pump (G1311C), standard autosampler (G1329B), thermostatted column compartment (G1316A), and fluorescence detector (G1321B). High-resolution mass spectral analysis was performed using an Agilent 1290-MS 6230 TOF-MS system in positive mode.

Valienamine and β-valienamine standards and the test samples were detected through pre-column derivatization by OPA and β- mercaptoethanol in H_3BO_3 buffer. Separation and quantification of the derivatives were performed on an Eclipse XDB-C18 (5 μm, 4.6 × 150 mm) column, and the samples were eluted with a gradient of acetonitrile at 30 °C and monitored by FLD.

3.5. Calibration and validation

Both the derivatization and separation conditions were optimized to determine an automatic online procedure. The asymmetry factor (As), resolution (R) and theoretical plate number (N) of the epimer standards were calculated to evaluate the suitability of the separation parameters such as peak shape and inter-sample separation ability. Selectivity, linearity, sensitivity, precision, and accuracy were investigated to validate the method.

4. Conclusions

This is the first demonstration of a separation procedure for valienamine and β-valienamine epimers through the use of a chiral derivatization reagent. The method was shown to be sensitive and reliable and a suitable necessary tool; it successfully helped in the construction of an asymmetric synthetic route generating valienamine and β-valienamine with high stereospecificity. It represents a promising strategy for chemical synthesis employing biological agents.

Disclosure statement

No potential conflict of interest was reported by the authors.

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