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Short communication

Quantitative analysis of valienamine in the microbial degradation of validamycin A after derivatization with *p*-nitrofluorobenzene by reversed-phase high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatography method for the quantitative analysis of valienamine in the microbial degradation of validamycin A, using a procedure for pre-column derivatization of valienamine with *p*-nitrofluorobenzene is described. Valienamine in the broth was first isolated with the ion-exchange method. The optimized conditions for the derivatization were the reaction time 30 min and reaction temperature 100 °C. With the mobile phases consisting of acetonitrile–water (12:88) (eluent A) and methanol (eluent B), the gradient was carried out with 100% of A for 15 min and then 100% of B for another 10 min. The parameters in the process were the flow rate of the mobile phase 1.0 ml/min, the injection volume 20 μl, the column temperature 40 °C and wavelength of ultraviolet detection 398 nm in all runs. A good linearity was found in the range of 0.5–150.0 μg/ml. Both intra- and inter-day precisions of valienamine, expressed as the relative standard deviation, were less than 9.4%. Accuracy, expressed as the relative error, range from −0.5 to 2.7%. The mean absolute recovery of valienamine at three different concentrations was 94.2%. The method was proved suitable for the study on the process of microbial degradation of validamycin A to produce valienamine.

Keywords: Derivatization, LC; Detection, LC; Valienamine; *p*-Nitrofluorobenzene; Validamycin A

1. Introduction

Valienamine (Fig. 1), intermediate named by the microbial degradation of validamycins by several soil bacterial such as *Pseudomonas denitrificans* [1] and *Flavobacterium saccharopinlurn* [2], or the *N*-bromosuccinimide cleavage of validoxylamine A or its derivatives [3,4], are pseudo-amino-sugar analogs of D-glucose, in which the ring oxygen is replaced with a carbon atom with the same configuration. Valienamine [(1S, 2S, 3S, 4R)-1-amino-5-(hydroxymethyl) cyclohex-5-ene-2,3,4-triol] has α-glucosidase inhibitory activity, inhibiting 50% activity of maltase and sucrase at a concentration of 3.4×10^{-4} and 5.3×10^{-5} M, respectively, and has showed antibiotic activity against *Bacillus species* [5–8].

Valienamine and its derivatives have been found to be key components for biological activities in pseudo-aminosugars and pseudo-oligosaccharides such as validamycins, acarbose (Glucobay) [9], amylostatins [10], adiposins [11], acarviosin [12], trestatins [13] and voglibose {1, *N*-[2-hydroxy-1-(hydroxymethyl)ethyl]valiolamine, AO-128, Basen}. These pseudo-oligosacchrides exhibit stronger enzyme inhibitory activities than valienamine itself. Some of them (acarbose and voglibose) are available in clinical use as antihyperglycemic agents. The production of these *N*-substituted branch-chain derivatives can be synthesized using valienamine as a starting material [14–19], especially acarbose and voglibose. Therefore, valienamine is one of the most important chemical intermediates for the production of glucosidase inhibitors, which are potential to be medicines.

Although valienamine can also be produced with chemical synthesis [20–29], its production with microbial degradation

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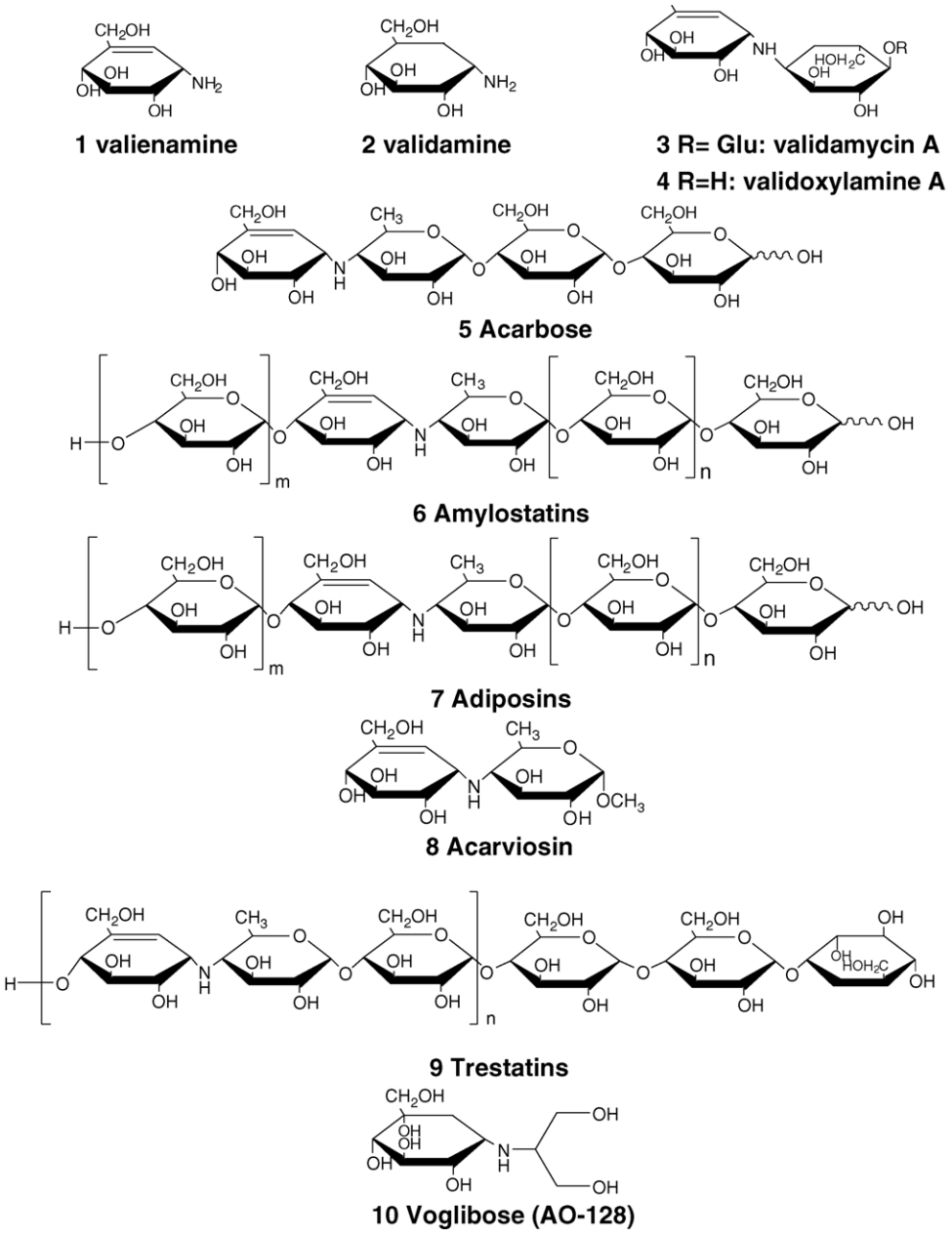

Fig. 1. Chemical structures of the valienamine related compounds.

is one of the most potent methods for commercial production. Now in China, validamycins are one of the largest yield antibiotics. The product has been exported to Southeast Asia and other countries. Its fermentation concentration reaches 30,000 μg/ml broth and its cost is very low. Therefore, the method of using validamycins as the substrate to produce valienamine is potential and hopeful in China.

Currently one of the most used analytical methods to indicate valienamine is thin layer chromatography; however, this technique does not provide an accurately quantitative measure. On the other hand, a quantitative analysis of valienamine is crucial to control and optimize the process of microbial degradation to produce valienamine. Here, a new method for the quantitative analysis of valienamine by reversed-phase high-performance liquid chromatography with UV detection is described. Before the sample flowed into the column, valienamine was derived with *p*-nitrofluorobenzene because the aqueous solution of valienamine does not show any characteristic absorption maximum in the region 200–360 nm except end absorption [30].

2. Experimental

2.1. Microorganism and its culture conditions

Microorganism used in the work was screened from the rice soil near Hangzhou, China by our laboratory and

kept on nutrient agar slants. The organism was grown in 500 ml shaking flasks containing 50 ml of nutrient medium (1.5% validamycin A, 0.5% yeast extract, 1.0% peptone, 0.5% NaCl, 0.7% K_2HPO_4, 0.3% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$ with natural pH) at 30 °C with rotating shaking (200 rpm) for 4 days.

2.2. Instrumentation chromatographic conditions

The chromatographic system (Shimadzu, Japan) consisted of two pumps (LC-10AT *VP*), an UV–vis detector (SPD-10A *VP*), a column oven (CTO-10AS *VP*) and an N-2000 workstation (Zhejiang University, China). Chromatography was performed on a Hypersil ODS2 column (250 mm × 4.6 mm i.d., particle 5 μm, Dalian Elite Analytical Instruments Co. Ltd., China). The gradient elution was carried out with acetonitrile–water (12:88) (eluent A) and methanol (eluent B). The gradient elution started at 100% of A for 15 min and then changed to 100% of B for another 10 min. The injection volume was 20 μl and the flow rate of the mobile phase was 1.0 ml/min in all runs. The column temperature was held at 40 °C and ultraviolet detection was performed at wavelength of 398 nm.

2.3. Reagents and materials

Valienamine was obtained by the cleavage of validamycin A according to Ogawa et al. [3,4]. The purity was more than 98%. The internal standard for validamine employed valienamine, too. *p*-Nitrofluorobenzene was purchased from Acros Organics (Belgium). HPLC grade methanol and acetonitrile were from Merk (Germany). Distilled water, prepared from deionized water, was used throughout the study. Other chemicals and solvents were all analytical grade. D113 resin was purchased from Purolite (China). Thin layer was Silica G (Qingdao Ocean Chemicals Co. Ltd., China).

2.4. Derivatization protocol

The aqueous solution of valienamine and validamine does not show any characteristic absorption maximum in the region 200–360 nm except end absorption. Therefore, valienamine was derivatized with *p*-nitrofluorobenzene to produce *N*-*p*-nitrophenyl-valienamine (**11**) (Fig. 2) before being detected by UV–vis detector according to Asano et al. [31] with modification.

2.5. Reaction with p-nitrofluorobenzene

The reaction with *p*-nitrofluorobenzene was performed in the test tubes containing the solid sample or the dried liquid sample together with 2.0 ml dimethylformamide solution of 1% *p*-nitrofluorobenzene and 1.5% triethylamine. After vigorous vortex, the tubes were further incubated for 10–35 min at 70–100 °C in a water bath. Before injection, the samples were diluted with 8 ml eluent A.

2.6. Preparation of standard samples and samples from microbial degradation of validamycins

The stock standard solution of valienamine was prepared by dissolving the accurately weighed valienamine in water to give a final concentration of 1.0 mg/ml. The solution was then successively diluted with water to achieve different standard working solution. Then 1.0 ml at the concentrations of 0.5–150.0 μg/ml were spiked into the test tubes, respectively, and dried in the oven at 105 °C.

At the end of culture, the broth was centrifuged to discard the cells and 5–40 ml of broth was passed through a column of D113 (NH_4^+ form, 20 ml), which was eluted with 100 ml 0.5 N aqueous ammonia. The eluate was concentrated under vacuum condition at the temperature of less than 65 °C to 10.0 ml. Then 0.1–1.0 ml of concentrate was put into a test tube and dried at 105 °C in the oven to get the sample.

2.7. Performance characteristics

2.7.1. Selectivity

The control blank broth was prepared from the culture of microorganism with validamycin A-free medium. The retention time of valienamine was typically 11.60–11.70 min. Variable peaks eluted between 7 and 10 min, however, this did not interfere with the analyte. No interfering endogenous components were observed in blank broth. The *p*-nitrofluorobenzene derivation of valienamine could be detected selectively in the broth.

2.7.2. Calibration and calculation procedures

Standard curves were constructed using weighted ($w = 1/c^2$) linear least-squares regression analysis of the observed peak area ratios of the derivatized products of valienamine and the internal standard. The unknown sample concentrations were calculated from the linear regression equation of the peak area ratio against concentrations of the calibration curve.

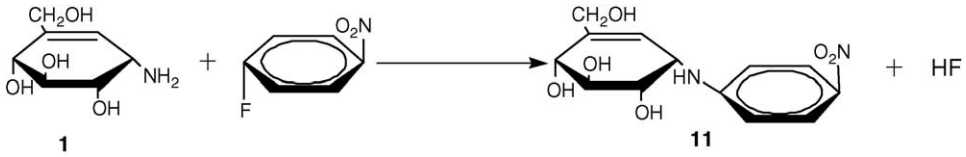

Fig. 2. Chemical reaction of valienamine with *p*-nitrofluorobenzene.

2.7.3. Precision and accuracy of the assay

In order to evaluate the intra-day precision and accuracy, six replicate samples of each concentration of QC samples were analyzed on the same day. The inter-day validation was evaluated on the QC samples of different days. Intra- and inter-day assays were assessed using six spiked degraded samples at each concentrations of 2.0, 40.0 and 120.0 μg/ml. The accuracy was evaluated as the relative error, and the precision was evaluated by the relative standard deviation.

2.7.4. Absolute recovery and analyte stability

The absolute recoveries of valienamine from the broth was determined as follows: the broth using validamycin A-free medium with added valienamine (2.0, 40.0 and 120.0 μg/ml). Absolute recovery was calculated by comparing the peak areas so obtained with those obtained by derivation of aqueous solution or broth.

2.7.5. Application of the analytical method

The established analytical method was used to investigate the profile of microbial degradation of validamycin A to produce valienamine. The broth concentration–time curves of valienamine were plotted.

3. Results and discussion

3.1. Pre-treatment of samples by D113 resin

5.0–40.0 ml centrifuged broth passed through a D113 (NH_4^+ form) column (20 ml). The results of the valienamine recovery are shown in Table 1. From Table 1, the volume of broth influenced the valienamine recovery greatly. If the volume was less than 20 ml, the recovery was greater than 97.0%. But when the volume was more than 20 ml, the recovery was decreased obviously. Therefore, 20 ml was selected in the following experiments with the same broth.

3.2. Optimal derivatization conditions

The optimal derivatization conditions were determined by investigating the effects of reaction temperature and time.

3.2.1. Influence of reaction temperature

By comparing peak areas of the derivatized product of valienamine, *N-p*-nitrophenyl-valienamine, under reaction time of 10 min, the influence of reaction temperature was observed (Fig. 3). The experiment showed that the peak areas of the *N-p*-nitrophenylvalienamine increased with the higher reaction temperature. Considering the convenient boiling water bath, 100 °C was chosen as the reaction temperature.

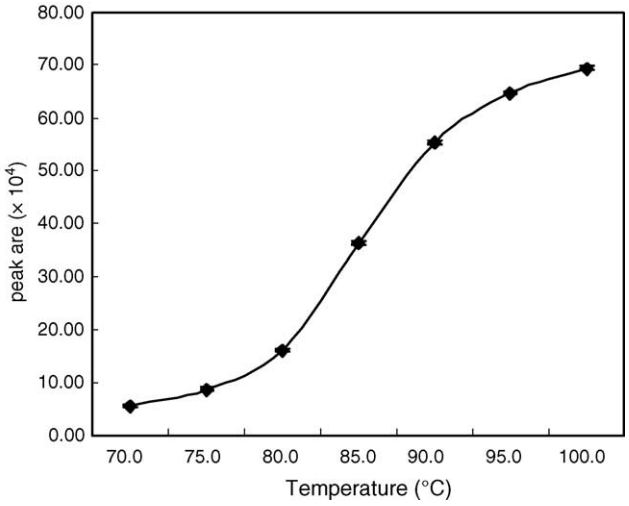

Fig. 3. Influence of reaction temperature on the peak area.

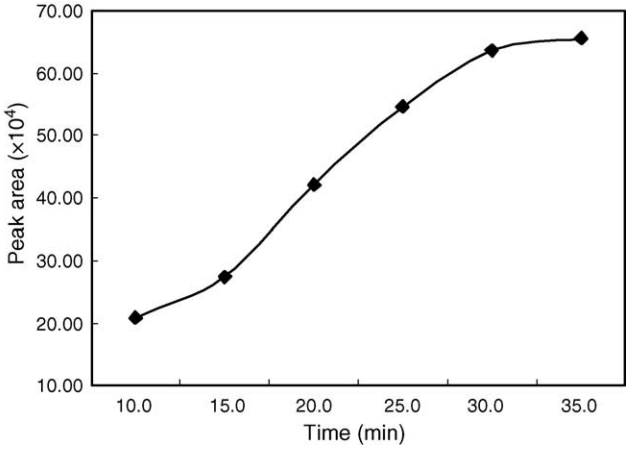

Fig. 4. Effect of reaction tome on the peak area.

3.2.2. Effect of reaction time

With the reaction temperature of 100 °C, the reaction time was investigated by comparing peak areas of *N-p*-nitrophenylvalienamine (Fig. 4). The peak areas increased with longer time. Above the reaction time of 30 min, no significant increase in the peak areas was observed. Therefore, 30 min was selected as the reaction time.

3.3. Optimization of the mobile phase

The mixtures of methanol or acetonitrile with water at various ratios were examined as mobile phase on C_{18}-column. When methanol existed or acetonitrile content was

Table 1
Influence of broth volume on the valienamine recovery in D113 pre-treatment[a]

Broth volume (ml)	5.0	10.0	15.0	20.0	25.0	30.0	35.0	40.0
Valienamine recovery (%)	99.7	102.1	100.6	97.8	92.9	85.6	73.1	60.4

[a] The experiments were repeated three times and the average was selected.

Table 2
Precision and accuracy for analysis of the valienamine in broth ($n = 6$)

Concentration spiked (μg/ml)	Intra-day concentration found (mean ± S.D.)	R.S.D. (%)	Inter-day concentration found (mean ± S.D.)	R.S.D. (%)	Accuracy R.E. (%)
2.0	1.93 ± 0.06	3.1	2.03 ± 0.04	2.0	2.7
40.0	40.33 ± 1.47	3.6	38.56 ± 2.03	5.3	−0.5
120.0	116.70 ± 11.06	9.4	118.70 ± 9.12	7.7	2.3

more than 20, the peaks of valienamine and validamine were seriously overlapped. At acetonitrile content of 12%, the peaks could be separated with good forms. But the retention time of valienamine increased with water content. The temperature of the column was changed to 30, 40 and 50. The retention time was decreased with the increase of column temperature. In order to get good form of peaks and improve the efficiency, 40 °C was employed as the column temperature. Under the optimized conditions of the mobile phase ratio and column temperature (as described above), a sharper peak was obtained. After the product peaks, methanol had been used to wash off other components because of the strong polarity of methanol. So a gradient program was employed.

3.4. Linearity of calibration curve and lower limit of quantification (LLOQ)

The linearity test of calibration range was carried out in the range of 0.5–150.0 μg/ml with standard samples. Calibration curves were linear in the studied range with good correlation coefficient ($r = 0.9981$). A typical linear regression equation

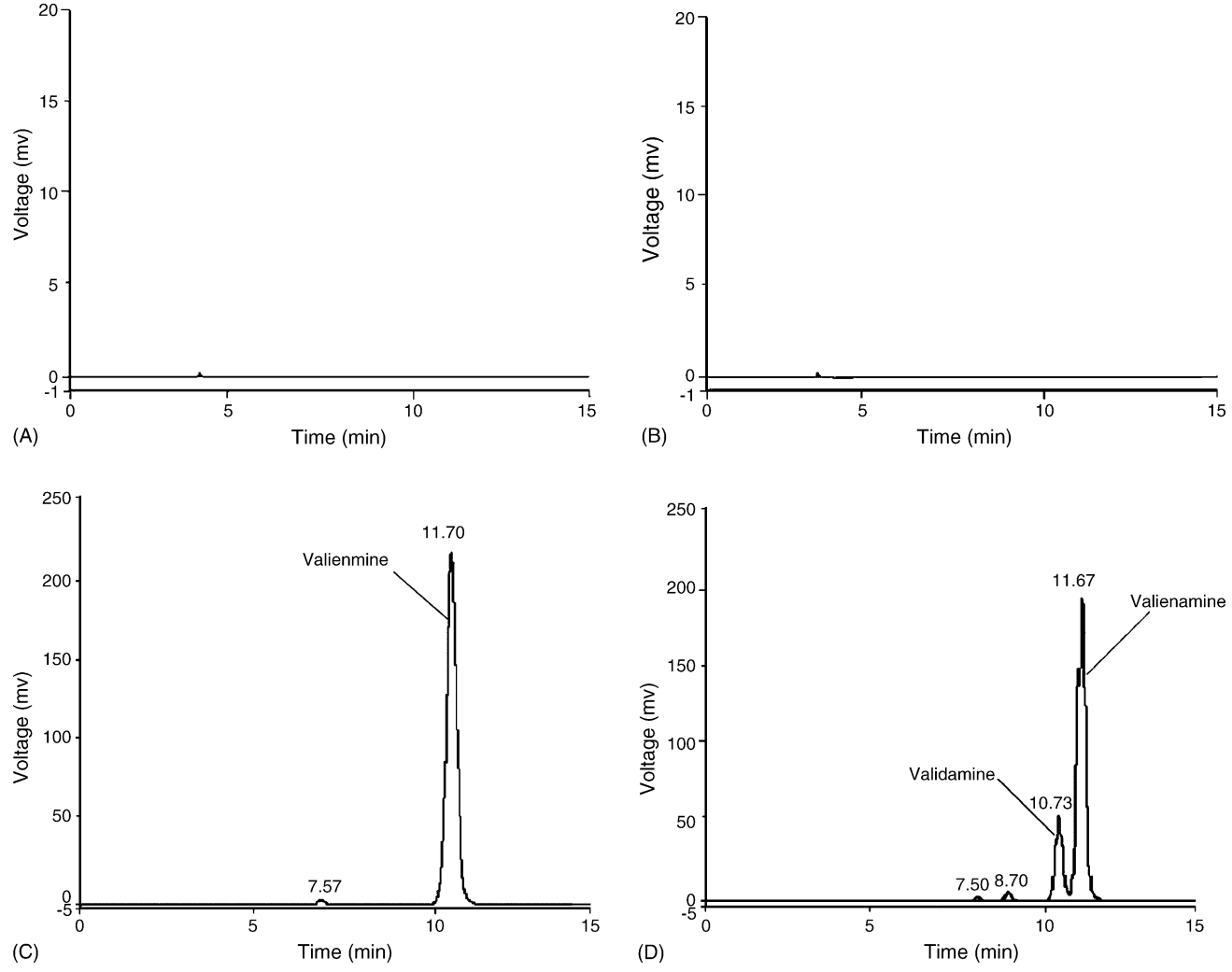

Fig. 5. Representative chromatography of vallenamine. (A) Chromatogram of blank sample (*p*-nitrofluorobezene without valienmine). (B) Chromatogram of blank sample (valienmine without *p*-nitrofluorobenzene). (C) Chromatogram of valienmine (100.0 μg/ml). (D) Sample of microbial degradation of valienmycin A (valienmine: 86.2 μg/ml).

for the corresponding curves was $y = 2 \times 10^{-5}x + 0.0046$, where x represents the peak area of valienamine, and y represents concentration of valienamine. Lower limit of quantification (LLOQ) was defined as the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision $\leq$15% and accuracy within $\pm$15% of the true concentration. LLOQ of valienamine was 0.5 μg/ml for the determination of valienamine in the broth.

3.5. Assay precision and accuracy

The intra- and inter-day precision and accuracy for valienamine from QC samples is summarized in Table 2. In the assay, the intra- and inter-day precisions ranged from 3.1 to 9.4 and from 2.0 to 7.7, respectively. The accuracy ranged from −0.5 to 2.7. These data suggested that the method was accurate and reproducible for the determination of valienamine in the broth.

3.6. Absolute recovery and analyte stability

The absolute recoveries of valienamine, at concentrations of 2.0, 40.0 and 120 μg/ml, were 90.4 ± 5.7, 91.9 ± 7.3 and 93.0 ± 6.2%, respectively. The absolute recovery of the external standard recovery was determined at the concentration used in the assay procedure and was found 94.2 ± 4.1%. Valienamine was found to be stable at the room temperature for at least half year. The derivatized product of valienamine and validamine, *p*-nitrofluorobenzenevalienamine and *p*-nitrofluorobenzenevalidamine, were also shown to be stable for at least 24 h at room temperature.

3.7. Chromatographic analysis

After selecting the optimal wavelength (at 398 nm), the typical chromatograms of derivatized product, *N*-*p*-nitrophenylvalienamine, is shown in Fig. 5. The peaks for their derivatized products were not interfered by endogenous substances (such as triethylamine and *p*-nitrofluorobenzene, their retention times were all after 15.0 min which were eluted by methanol). The retention time for derivatized product valienamine was approximately 11.70 min. From Fig. 5, the peaks of valienamine (11.70 min) and validamine (10.73 min) could be totally separated.

At the end of fermentation with 1.5% validamycin A as the substrate, valienamine concentration in the broth was 2063.8 μg/ml broth. As from the chromatograms, the peak of valienamine is higher than that of validamine. With valienamine as the internal standard for validamine, the concentration of validamine was 526.0 μg/ml broth.

4. Conclusion

In this paper, we present a reverse-phase HPLC procedure, which was used to resolve and quantify valienamine, an important chemical intermediate for medicines, in the broth of microbial degradation of validamycin A. The developed analytical method was sensitive to control the biotechnological process. After the optimization of derivatization conditions and chromatographic conditions, the linear calibration curve was obtained in the concentration range of 0.5–150.0 μg/ml with good correlation coefficient ($r = 0.9981$). The selectivity, precision, accuracy, recovery and stability of the method were investigated, which proved the method suitable for quantitative assay of valienamine in the microbial degradation of validamycin A.

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