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Short communication

High-performance liquid chromatographic determination of α-aminonitrile enantiomers after derivatization with *o*-phthalaldehyde and chiral thiols

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Abstract

o-Phthalaldehyde in combination with chiral thiols is described as a chiral reagent for the resolution of the enantiomers of α-H-α-aminonitriles and α-alkyl-α-aminonitriles. Separation of the resulting diastereomers was carried out by reversed-phase chromatography and the derivatives were detected at UV 340 nm. The identity of the diastereomeric derivatives was confirmed by LC–MS. Among the chiral thiols tested, *N*-acetyl-D-penicillamine and β-mercaptoisobutyric acid gave the best resolution for the α-aminonitriles studied. For quantitative enantiomeric excess determination, β-mercaptoisobutyric acid was chosen as this thiol could be obtained in high enantiomeric purity from a commercial source. The rate of the reaction of various α-aminonitriles with *o*-phthalaldehyde and β-mercaptoisobutyric acid was studied. The accuracy of the method was investigated by a comparison of theoretical and measured enantiomeric excess.

Keywords: Enantiomer separation; Molecular modelling; Derivatization, LC; α-Aminonitrile

1. Introduction

Enantiopure α-aminonitriles are important chiral building blocks for amino acids. Several enantioselective routes towards α-aminonitriles have been described, such as enzymatic resolutions using Pen G acylase [1] and lipase [2], diastereomeric resolution [3] and asymmetric transformation [4]. In conjunction with these routes, analytical methods are required to monitor the enantiomeric purity of the α-aminonitriles. HPLC is an efficient and therefore widely used technique for accurate enantiomeric analysis. HPLC methods for the resolution of optical isomers of amino compounds include direct and indirect approaches in reversed- and normal-phase mode. Regarding α-aminonitriles, direct HPLC methods in reversed-phase mode [5] and normal-phase mode [6] have been described for the enantiomeric separation of some aromatic α-aminonitriles. As we were studying enantioselective routes towards both α-H-α-aminonitriles and α-alkyl-α-aminonitriles in aqueous media, we initially evaluated a direct reversed-phase HPLC method [5]. This method, however, was unsuitable for the separation of α-alkyl-α-aminonitriles. As our goal was to employ a generic method for the enantiomeric analysis of our α-aminonitriles, we extended our search to include indirect HPLC methods. Among the most popular indirect HPLC methods for the enantiomeric analysis of compounds bearing a primary amino function are reversed-phase methods in which *o*-phthalaldehyde (OPA) in combination with a chiral thiol is used as a chiral reagent [7]. The applicability of the OPA chiral thiol approach was first demonstrated for protein amino acids [8,9]. Since that time, several classes of primary amines have been resolved, e.g. α-amino acid amides [10–13], α-amino carboxylic esters [10], β-amino alcohols [13–16], α-hydroxymethyl-α-amino

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acids [17,18], arylalkylamines [19], α-alkyl-α-amino acids [10–13,17,20] and nucleoamino acids [21]. In the majority of applications, N-acylated cysteines are used as chiral thiol in the OPA reaction. Other chiral thiols used are: thiosugars [16,22,23], neomenthylthiol [24], captopril [25], and 3-mercapto-2-methylpropionic acid [13]. No reference has been found to the enantiomeric analysis of α-aminonitriles by the OPA/chiral thiol approach.

The aim of this study was to evaluate the use of OPA in combination with several chiral thiols as reagents for the determination of the enantiomers of α-H-α-aminonitriles and α-alkyl-α-aminonitriles by reversed-phase HPLC of the corresponding diastereomeric isoindolyl derivatives.

2. Experimental

2.1. Materials

All α-aminonitriles (Fig. 1A) were synthesized according to general procedures [26]. *N*-Acetyl-D-penicillamine (NAP) was obtained from Fluka (Buchs, Switzerland). *N*-Isobutyryl-L-cysteine (NIBC) was purchased from Novabiochem (Läufelfingen, Switzerland). D-*S*-Acetyl-3-mercapto-2-methylpropionic acid (MMPA) (name according to supplier: D-*S*-acetyl-β-mercaptoisobutyric acid), *N*-Acetyl-L-cysteine (NAC) and *o*-phthalaldehyde were purchased from Acros (Geel, Belgium). HPLC-grade methanol was supplied by Merck (Darmstadt, Germany). Water was purified with a Millipore (Bedford, MA, USA) Milli-Q system. All other chemicals were of analytical-reagent grade.

2.2. Instrumentation

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, USA) Model 1090 liquid chromatograph and a Gilson (Villiers-le-Bel, France) Model 231-401 autosampling injector for derivatization and injection. The injection loop had a capacity of 20 μl. The column used was a Nucleosil-120-C18 column (125 mm × 4.0 mm i.d., 5 μm) from Macherey-Nagel (Düren, Germany). The flow rate was 1 ml min^{-1} and the analysis was performed at ambient temperature. The derivatives were monitored with a Hitachi (Tokyo, Japan) Model F-1000 fluorescence detector using an excitation wavelength of 340 nm and an emission wavelength of 420 nm, and a Thermo Separations (Fremont, CA, USA) Spectrasystem UV2000 detector at 340 nm.

LC–MS analysis was performed on an API 150EX LC–MS system with a turbo ion interface in the negative ionization mode (Applied Biosystems, Nieuwerkerk a/d IJssel, Netherlands). The HPLC conditions used for LC–MS were the same as those used for LC–UV except that before entering the MS ionization interface the flow was split 1:5. The isoindolyl derivatives were measured as $[M - H]^-$ ion.

2.3. Eluent, reagent and derivatization procedure

The mobile phase consisted of 20 mM acetic acid, titrated to pH 6.0 with triethylamine. The concentration of methanol in the mobile phase is indicated in Figs. 2 and 4 and Table 1.

The buffer used in the derivatization procedure consisted of 50 mM phosphoric acid, titrated to pH 7.0 with sodium hydroxide.

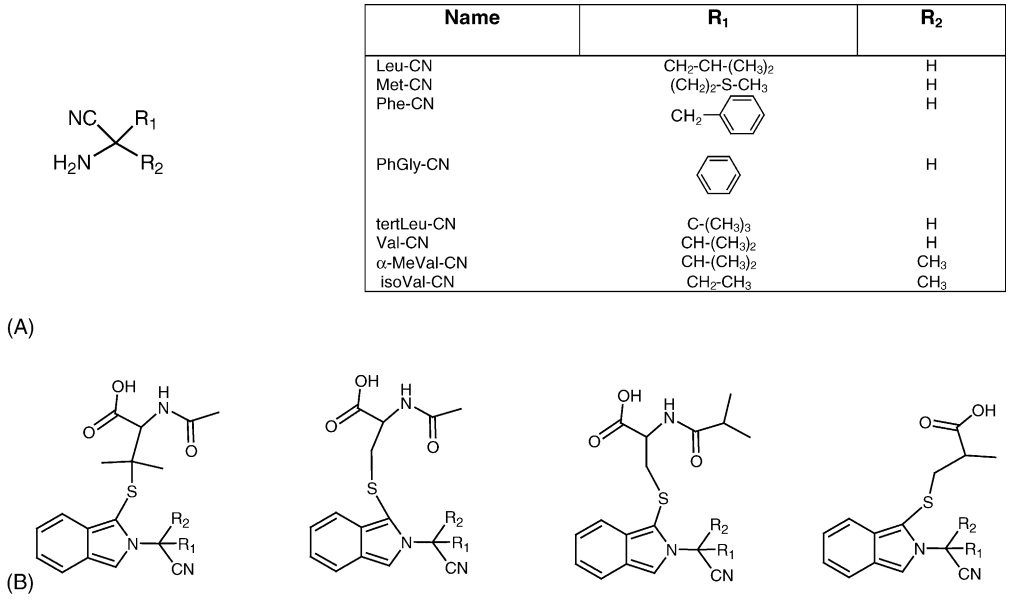

Fig. 1. (A) Structures of α-aminonitriles studied (*remark*: instead of the IUPAC names, the three-letter abbreviation of the corresponding amino acid followed by CN is used in the figures, table and text to denote the α-aminonitriles). (B) Proposed structures for the diastereomeric adducts formed in the reaction of Val-CN with (I) OPA/NAP, (II) OPA/NAC, (III) OPA/NIBC and (IV) OPA/MMPA.

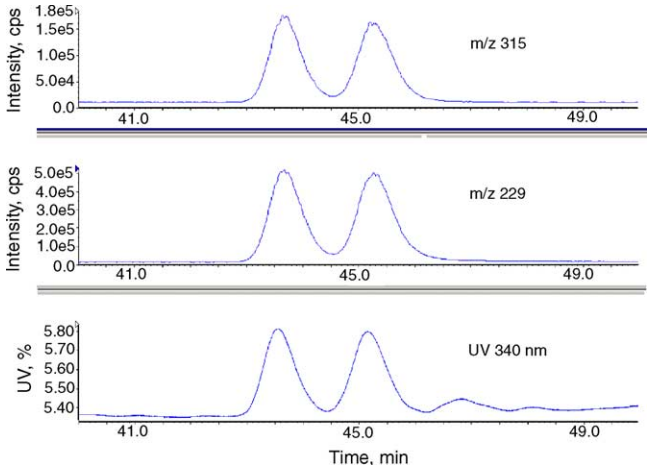

Fig. 2. Selected ion chromatograms (*m*/*z* 315 and *m*/*z* 229) and UV chromatogram of OPA/MMPA derivatives of racemic *iso*Val-CN. Column, 250 mm × 4.0 mm i.d. Nucleosil-120-C18 (3 μm); mobile phase, 20 mM triethylammonium acetate (pH 6.0)–methanol (70:30, v/v); flow rate 0.6 ml min^{-1}. For other conditions, see Section 2.

For the OPA reagent, a 0.23 M solution was prepared in methanol. The MMPA reagent was prepared by dissolving 50 mg of D-*S*-acetyl-3-mercapto-2-methylpropionic acid per ml of 1 M sodium hydroxide solution. The solution was stirred for 10 min at room temperature. To the MMPA solution thus obtained, 665 μl of buffer (pH 7.0) and 375 μl of 1 M HCI were added per ml of solution. For the other thiols used in this study, 160 mM solutions were prepared in a buffer (pH 7.0), except for NAP, which was dissolved in methanol. For the α-aminonitriles studied, 6 mM solutions were prepared in a buffer (pH 7.0).

Derivatization was performed automatically with a Gilson Model 231-401 system. The following volumes were mixed, in order of listing: 400 μl of OPA reagent, 50 μl of thiol reagent, and 250 μl of sample solution. After 15 min at room temperature, an aliquot of the reaction mixture was injected into the chromatographic system.

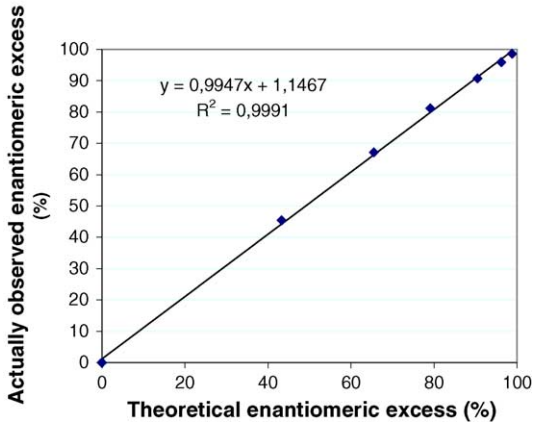

Fig. 3. Plot of theoretical enantiomeric excess vs. actually observed enantiomeric excess for Phe-CN. Reagent: OPA/MMPA.

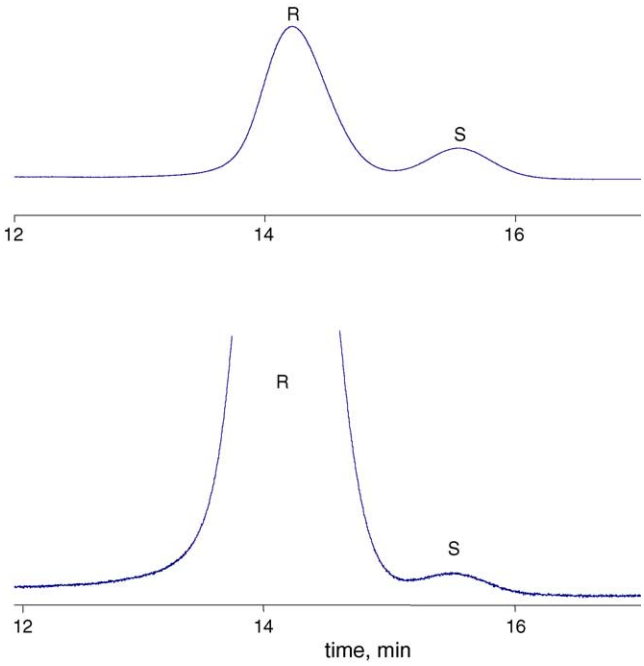

Fig. 4. UV chromatograms of OPA/MMPA derivatives of non-racemic Phe-CN. Upper chromatogram: enantiomeric excess 45.0%; lower chromatogram: enantiomeric excess 96.0%. The percentage of methanol in the mobile phase was 57% (v/v). Flow rate 0.6 ml min^{-1}. For other conditions, see Section 2.

Eluents for the pH study of the OPA/MMPA adduct of Leu-CN were prepared from a solution of 50 mM phosphoric acid, titrated to pH 7.0 with sodium hydroxide. Eluents of pH 2.3, 3.9 and 6.0 were prepared by titrating the sodium phosphate buffer solution (pH 7.0) with phosphoric acid. After pH adjustment, all four buffers were mixed with methanol (buffer/methanol 43/57%, v/v).

3. Results and discussion

Usually the reaction of OPA/thiol with amino compounds is performed at basic pH. However, at pH > 9, α-aminonitriles are irreversibly hydrated into their corresponding amides [27]. Despite the fact that this conversion does not influence the stereoconfiguration of the native α-aminonitriles, the accuracy of the determination is low as α-amino amides are often present as impurities in α-aminonitrile samples. For this reason, the derivatization reaction was performed at neutral pH.

In the literature, several derivatization protocols are reported for the conversion of amino compounds with OPA/thiol reagent. Protocols have been described in which OPA is used in excess compared with the thiol [12], in other studies [28–31] the thiol is used in excess compared with OPA. In our study, we tested both derivatization protocols to see whether the same derivative was formed upon reaction of α-aminonitriles with OPA/thiol. In one case, OPA/MMPA/Val-CN molar ratios were 55/5/1, and in the

Table 1
Retention factors (k') and selectivities (α) of OPA/MMPA, OPA/NAP, OPA/NIBC and OPA/NAC derivatives of α-aminonitriles

Compound	Reagent							
	OPA/MMPA		OPA/NAP		OPA/NIBC		OPA/NAC	
	k'_1[a]	α	k'_1[a]	α	k'_1[a]	α	k'_1[a]	α
Met-CN	3.18	1.12	2.23	1.21	3.17	1.00	1.35	1.00
Val-CN	3.46	1.18	2.59	1.19	3.48	1.11	1.50	1.08
PhGly-CN	5.54	1.04	4.74	1.12	5.46	1.06	2.34	1.02
*tert*Leu-CN	5.90	1.19	4.27	1.21	5.86	1.22	2.58	1.20
Leu-CN	6.06	1.20	4.24	1.21	6.02	1.06	2.66	1.10
Phe-CN	5.06	1.09	4.03	1.17	5.42	1.09	2.36	1.11

The percentage of methanol in the mobile phase was 57% (v/v). For other conditions, see Section 2.
[a] Retention factor of the first-eluted diastereomer.

other case, molar ratios were 20/60/1. After reaction for 10 min at pH 7.0, the same derivative peaks were obtained by HPLC in both cases. The only difference noticed between the two derivatization protocols was the fact that the formation rate of the derivatives was higher for the 20/60/1 mole ratio. This can be explained by the higher molar excess of the reagent (20-fold) in comparison to the 55/5/1 mole ratio, where the reagent excess is only five-fold. For further study, the 55/5/1 mole ratio (as described in Section 2) was used as the derivatives formed showed good solubility in this reaction mixture.

The proposed structures for the diastereomeric adducts of Val-CN and OPA/thiol formed at neutral pH are given in Fig. 1B.

With respect to the detection mode, it was noticed that in both of the above-mentioned derivatization protocols the UV response of the isoindolyl derivatives of the α-aminonitriles studied at 340 nm was greater than the fluorescence response at optimal excitation and emission wavelengths. For example, for the OPA/NAP derivative of Val-CN, the signal-to-noise ratio for UV detection at 340 nm was a factor of 27 higher than in the case of fluorescence detection at an excitation wavelength of 340 nm and an emission wavelength of 400 nm. Compared to the fluorescence response of thio-substituted isoindoles formed from amino acids, the fluorescence response of analogous derivatives from α-aminonitriles is substantially lower. This effect may be caused by the electron-withdrawing properties of the cyano group. The higher response for amino acids is observed when the isoindolyl derivatives are measured under eluent conditions whereby the acid function is dissociated (creating an electron-donating effect). Under conditions where the carboxylic group is undissociated (electron-withdrawing effect), a decrease in fluorescence is observed. Because of the enhanced sensitivity of the UV method, the diastereomeric derivatives of α-aminonitriles have been monitored in this mode.

α-Aminonitriles of different structures were selected in order to examine the effect of the chemical structures of the thiol reagents on the separation factors of the diastereomeric isoindolyl derivatives formed with the α-aminonitriles (Table 1) (it is understood in this context and in the following discussion that it is actually the diastereomeric isoindolyl derivatives formed from α-aminonitriles that are separated by HPLC). It was found that MMPA and NAP proved to be the only chiral selectors for which separation could be achieved for all α-aminonitrile enantiomers studied. With respect to the separation factors achieved with MMPA and NAP, it was observed that the aromatic α-aminonitriles, i.e. PhGly-CN and Phe-CN, showed lower selectivities than the aliphatic α-aminonitriles tested. For the OPA/MMPA derivatives of α-aminonitriles, the diastereomers of each compound could be baseline separated within 10 min.

To further extend the applicability of the new method, two α-alkyl-α-aminonitriles were tested with the OPA/MMPA method, i.e. *iso*Val-CN and α-MeVal-CN. The diastereomeric derivatives of both compounds could be separated (α = 1.04 for *iso*Val-CN and α = 1.06 for α-MeVal-CN). Comparison of the separation factor of α-MeVal-CN with its α-H analogue, i.e. Val-CN, shows that the presence of the α-methyl substituent leads to a substantial decrease in enantioselectivity.

To confirm the peak identity of the diastereomeric adducts of the α-aminonitriles studied, LC–MS analysis was performed. For all racemates separated, molecular weights were found that corresponded to the structures given in Fig. 1B.

To illustrate the chromatographic resolution obtained for the OPA/MMPA adduct of *iso*Val-CN, representative chromatograms in both UV and MS mode are shown in Fig. 2. Mass spectrometric characterization of the OPA/MMPA adduct of *iso*Val-CN was performed using the selective ion mode on the $[M - H]^-$ ion, i.e. m/z 315, and on the $[M - H–MMPA]^-$ ion, i.e. m/z 229. In this way, the identity of the UV peaks was confirmed.

The rates of derivative formation of OPA/NAP and OPA/MMPA with Val-CN and Phe-CN were studied as a function of the reaction time. For both reagents, maximum absorbance of the diastereomeric derivatives was reached after a reaction time of 10–15 min. To exclude possible kinetic resolution effects at reaction times <15 min, i.e. before reaching the plateau value, a fixed reaction time of 15 min was used in the automatic derivatization procedure for α-aminonitriles.

For accurate enantiomeric excess analysis, knowledge of the enantiomeric purity of the chiral thiol reagent is important. Since the suppliers of the two best performing thiols,

i.e. MMPA and NAP, were unable to supply this information, the enantiopurity of the thiols was measured using the "reverse" OPA reaction with L-Val (optical purity 99.9%) as chiral selector, as described earlier [13]. The values obtained for MMPA and NAP were 98.8% and 95.1%, respectively. As MMPA shows the highest enantiomeric purity, this thiol was used for further enantiomeric excess analysis.

For all racemic α-aminonitriles studied, the areas of the corresponding diastereomeric pairs were compared. When the peak area of the larger diastereomeric peak was set to 100%, the area of the other diastereomeric peak was found to be >98% in all cases. This difference was considered not significant and quantification was therefore based on calibration curves for the racemate.

The accuracy of the method, using the OPA/MMPA reagent, was checked by determining the enantiomeric excess of enantiomeric mixtures of Phe-CN, prepared by weighing amounts of both the *R* and the racemic forms. The results are given in Fig. 3. Linear regression analysis indicated that the correlation coefficient was 0.9991.

To illustrate the applicability of the method, representative chromatograms of the OPA/MMPA derivative of Phe-CN at 72.5:27.5 and 98:2 *R*/*S*-ratio are shown in Fig. 4. The elution order is *R* before *S*. The same elution order was found for the OPA/MMPA derivative of Val-CN. For the other α-aminonitriles studied, no elution order can be given as non-racemic standards were not available.

The detection limit at UV 340 nm for the first-eluting OPA/MMPA derivative of Phe-CN, based on a signal-to-noise ratio of 3, was 15 ng.

In this study we showed that the OPA/chiral thiol approach can be used for the enantiospecific analysis of α-H-α-aminonitriles and α-alkyl-α-aminonitriles. With respect to the mechanism of the diastereomeric separation, we showed in a previous study [32] that the formation of intramolecular hydrogen bonds between the thiol and amino acid residues in the diastereomeric isoindolyl adducts is an important factor in obtaining good selectivity. To check whether intramolecular hydrogen bonding between the cyano function of an α-aminonitrile and the COOH function of MMPA occurred in solution for the derivatives studied, separation factors were measured for the OPA/MMPA adduct of racemic Leu-CN using eluents at pH 2.3, 3.9, 6.0 and 7.0. At all pH values no significant change of the separation factor was observed ($\alpha = 1.20 \pm 0.01$). With respect to the retention factors, an increase was observed when going from pH 7.0 ($k'_1 = 7.3$) towards pH 2.3 ($k'_1 = 33.8$), which may be explained by protonation of the carboxylate anion of MMPA at acidic pH (calculated pK_a value [33] of MMPA is 1.8), resulting in a more strongly retained compound. If intramolecular hydrogen bonding was involved, one would expect an increase in the separation factor at pH 7.0 (in comparison to pH 2.3) as it is known that hydrogen bonds involving a carboxylate ion are stronger than those involving carboxylic acids. As no change of α occurs in the pH region 2.3–7.0, it is therefore very unlikely that intramolecular hydrogen bonding between the carboxylic function of MMPA and the cyano function of the α-aminonitrile is involved in the derivatives studied.

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