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RESOLUTION OF D- AND L-AMINO ACIDS AFTER PRECOLUMN DERIVATIZATION WITH o-PHTHALALDEHYDE BY MIXED CHELATION WITH Cu(II)–L-PROLINE*

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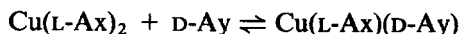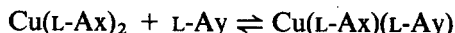(Received November 4th, 1985)

SUMMARY

Highly selective separation of amino acids can be accomplished by reversed phase chromatography of mixed chelate complexes of the analyte acids with equimolar concentrations of Cu(II) and an optically active second amino acid in the mobile phase. Resolution of D- and L-Dns-amino acids was accomplished this way using L-proline or L-histidine as the second amino acids. We have now extended the mixed chelation approach to resolve o-phthalaldehyde (OPA) derivatives. The free amino acid was reacted with OPA in the presence of N-acetyl-L-cysteine. High-performance liquid chromatography of the derivative was then performed on a reversed-phase column, with a mobile phase containing L-proline and Cu(II), using fluorescence detection. All primary amino acids reacted rapidly with OPA without measurable side products. The optical isomers were resolved. Study of the mechanism of optical selectivity confirmed that the chiral sulfhydryl reagent was responsible for the formation of diastereomeric mixed chelate complex and for the resolution of the isomers. The same approach was also applied to resolve stereoisomers of amines and sulfhydryl compounds.

INTRODUCTION

Methods for separating D- and L-Dns-amino acids by reversed-phase high-performance liquid chromatography (HPLC) of mixed chelate complexes of analyte amino acids formed by reaction with a chiral Cu(II) complex in the mobile phase were previously described[1-7]. Enantiomers are resolved based on a chiral Cu(II) complex in the mobile phase with which D- and L-amino acids can form 2 diastereomeric ternary complexes of different stability:

$$Cu(L\text{-}Ax)_2 + L\text{-}Ay \rightleftharpoons Cu(L\text{-}Ax)(L\text{-}Ay)$$

$$Cu(L\text{-}Ax)_2 + D\text{-}Ay \rightleftharpoons Cu(L\text{-}Ax)(D\text{-}Ay)$$

The system separates the different amino acids one another along with separating their optical isomers.

Many approaches to the separation of D- and L-amino acids by metal complexation have also been reported. Karger and co-workers[8,9] used L-2-alkyl-4-octyl-diethylenetriamine complexes of zinc and other metals in the mobile phase to separate dansyl derivatives of amino acids. Hare and Gil-Av, who previously reported using Cu(II)–proline eluents[10] to separate free D-and L-amino acids, more recently studied Cu(II)-di-N-propyl-alanine[11]. Nimura *et al.*.[12] also resolved free amino acids on a reversed-phase column by adding Cu(II) complex of N-(*p*-toluene-sulfonyl)-L-phenylalanine. Grushka and co-workers[13,14], using Cu(II) complexes of aspartame and derivatives of aspartic acid resolved a number of D- and L-amino acids.

o-Phthalaldehyde (OPA) has been used as a post-column derivatization agent for the detection of amino acids for many years[15]. Recently, OPA was also used as a pre-column derivatization agent for the analysis of amino acid by reversed-phase chromatography[16,17]. However, the optical isomers were not resolved in these separations. In this paper, we extended the mixed chelation approach for the separation of D- and L-amino acids to the OPA derivatives. The DL-amino acids were derivatized with OPA in the presence of a chiral sulfhydryl reagent, N-acetyl-L-cysteine (NAC). HPLC of the derivatives was then performed on a reversed-phase column using fluorescence detection. The optical isomers were separated as the diastereomeric mixed chelate Cu(II)–L-proline complexes. Study of the mechanism of stereoselectivity confirmed that the chiral sulfydryl reagent was responsible for forming the mixed chelate complex. The observation stimulated us to resolve also optical isomers of amines and sulfhydryl compounds with the present approach.

EXPERIMENTAL

Reagents

Acetonitrile distilled in glass was bought from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), and *o*-phthalaldehyde, N-acetyl-L-cysteine (NAC) and the free amino acids from Sigma (St. Louis, MO, U.S.A.). The mobile phases in general contained 10–15% of acetonitrile in a buffer that was $5.0 \cdot 10^{-3}$ M of L-proline, $2.5 \cdot 10^{-3}$ M of copper sulfate and 2.0 g of ammonium acetate, pH 7.0.

Instrumentation

The chromatograph consisted of two Altex 110A pumps and a Model 420 gradient microprocessor (Altex Scientific, Berkeley, CA, U.S.A.); a Rheodyne 7105 injection valve and an analytical column; 15 × 0.42 cm, packed with Nucleosil 5 C_{18} by the downward slurry technique. The OPA-amino acids were detected with a Fluoro-tec filter fluorometer (American Research Products, Kensington, MD, U.S.A.). The amplified detector signals were read out on a Model 4416 data system (Nelson Analytical, Cupertino, CA, U.S.A.) and a Model 56 chart recorder (Perkin Elmer, Norwalk, CT, U.S.A.).

Derivatization procedure

The OPA–NAC reagent was prepared by dissolving 20 mg of *o*-phthalaldehyde and 24 mg of N-acetyl-L-cysteine in 6 ml of methanol–water (50:50). Derivatization

TABLE I

CAPACITY RATIO (k') AND SELECTIVITY (α) OF AMINO ACIDS DERIVATIZED WITH o-PHTHALALDEHYDE IN THE PRESENCE OF N-ACETYL-L-CYSTEINE

Mobile phase: 2.5 mM Cu(II)–L-proline with no acetonitrile for 1–5, 5% acetonitrile for 6–9, and 10% acetonitrile for 10–18.

Amino acid	k'_L	k'_D	α
1 Asp	1.9	2.5	1.3
2 Glu	5.8	9.6	1.6
3 Ser	17.8	24.3	1.4
4 Asn	22.4	31.0	1.4
5 Thr	48.6	58.7	1.2
6 Lys	8.2	12.4	1.5
7 Cit	8.6	13.8	1.6
8 Ala	11.5	15.6	1.4
9 Arg	17.0	22.9	1.3
10 Tyr	6.2	9.8	1.6
11 Val	9.0	16.5	1.8
12 Met	16.3	19.7	1.2
13 Nval	17.2	22.2	1.3
14 Etho	37.2	45.7	1.2
15 Try	39.9	60.1	1.5
16 Phe	43.0	48.2	1.1
17 Leu	48.4	65.4	1.4
18 Nleu	48.6	62.1	1.3

of the free amino acids was accomplished in a vial by dispensing 300 μl of 1.0 M sodium borate buffer pH, 9.4, 50 μl of the OPA–NAC reagent and 5 μl of a stock solution of the amino acid. After mixing and standing at room temperature for five minutes, a 50-μl aliquot of the reaction mixture was injected into the chromatograph.
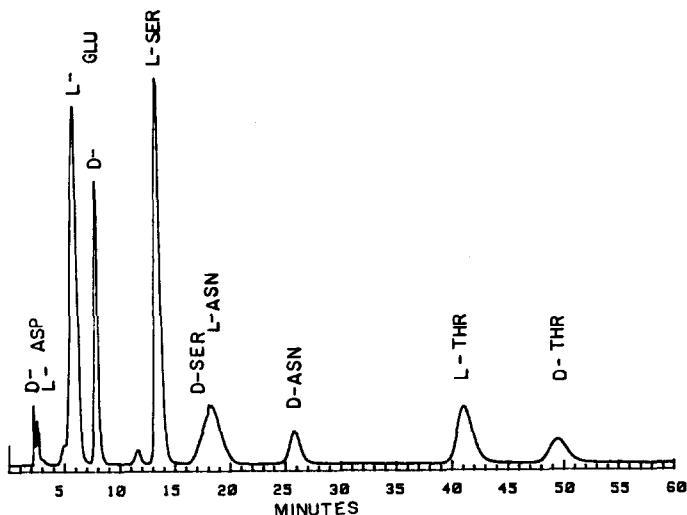

Fig. 1. Chiral separation of OPA derivatives of polar amino acids. Mobile phase: 5 mM L-proline, 2.5 mM CuSO$_4$ · 5H$_2$O and 2.0 g ammonium acetate, pH 7.0. Flow-rate, 2.0 ml/min.

RESULTS

The D- and L-isomers of amino acids were separated as the OPA–NAC derivative on a reversed-phase column using mobile phases containing Cu(II)–L-proline and varying amounts of acetonitrile (Table I and Figs. 1–3). As in reversed-phase systems, lowering the concentration of acetonitrile in the mobile phase improves the selectivity and resolution (Tables II and III).

Amino acids derivatized by the more conventional method with OPA and mercaptoethanol were also separated on the reversed-phase column under identical chromatographic conditions. These amino acid derivatives from the OPA-mercaptoethanol reaction, although more hydrophobic with large k' values, were not optically resolved (Table IV). The observation suggests that N-acetyl-L-cysteine is responsible for mixed chelation with Cu(II)–L-proline and the analyte amino acid is not. Since mixed chelaton is independent of the analyte, the present chromatographic technique is applicable to chiral amines which also react with the OPA–NAC reagent. The separation of D- and L-normethanephrine is shown (Fig. 4). N-Acetylpenicillamine is a sulfhydryl amino acid which can substitute N-acetyl-L-cysteine in the OPA reaction. Optical isomers of N-acetylpenicillamine were resolved as the sulfhydryl agent when reacted with OPA with L-arginine supplied as the source of chiral amine (Fig. 5).

DISCUSSION

In a continuing effort to resolve enantiomers, we have extended the mixed chelation approach to the OPA derivatives of amino acids. It has long been known
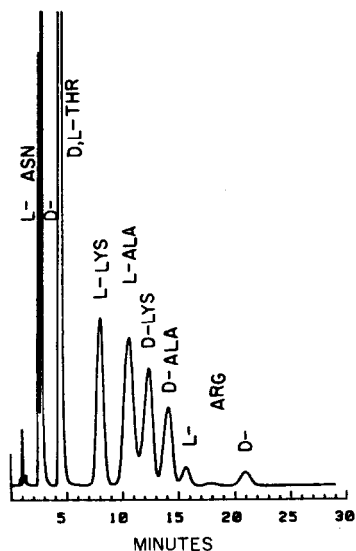

Fig. 2. Chiral separation of OPA derivatives of amino acids. Mobile phase: 5.0% acetonitrile in a buffer containing 5 mM L-proline, 2.5 mM CuSO$_4$ · 5H$_2$O and 2.0 g ammonium acetate, pH 7.0. Flow-rate 2.0 ml/min.
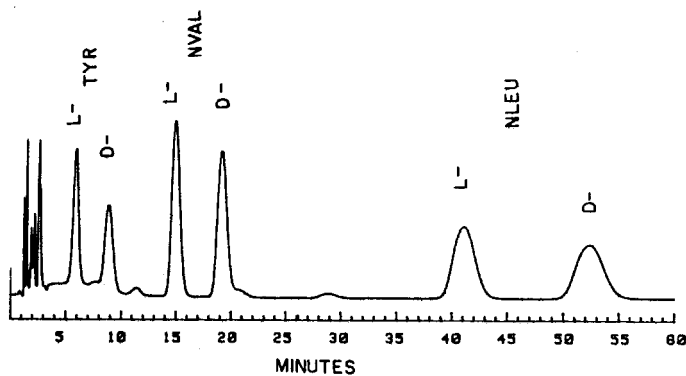
Fig. 3. Chiral separation of OPA derivatives of tyrosine, norvaline and norleucine. Mobile phase: 10% acetonitrile in a buffer containing 5 mM L-proline, 2.5 mM CuSO$_4$ · 5H$_2$O and 2.0 g ammonium acetate, pH 7.0. Flow-rate 2.0 ml/min.

TABLE II

CAPACITY RATIO (k') AND SELECTIVITY (α) AS A FUNCTION OF ACETONITRILE CONCENTRATION

Amino acids	0% acetonitrile			5% Acetonitrile		
	k' (L)	k' (D)	α	k' (L)	k' (D)	α
Asp	1.9	2.5	1.3	—	—	—
Glu	5.8	9.6	1.6	0.8	0.8	1.0
Ser	17.8	24.3	1.4	—	—	—
Asn	22.4	31.0	1.4	1.9	2.3	1.2
Thr	48.6	58.7	1.2	3.8	4.0	1.0
Ala	—	—	—	11.5	15.6	1.4

TABLE III

CAPACITY RATIO (k') AND SELECTIVITY (α) AS A FUNCTION OF ACETONITRILE CONCENTRATION

Amino acid	10% Acetonitrile			12.5% Acetonitrile		
	k' (L)	k' (D)	α	k' (L)	k' (D)	α
Tyr	6.2	9.8	1.6	2.8	3.5	1.3
Val	9.0	16.5	1.8	4.0	4.3	1.1
Met	16.3	19.7	1.2	7.3	9.1	1.3
Nval	17.2	22.2	1.3	7.6	10.1	1.3
Eth	37.2	45.7	1.2	15.2	18.2	1.2
Try	39.9	60.1	1.5	17.2	23.8	1.4
Phe	43.0	48.2	1.1	22.3	23.6	1.1
Leu	48.4	65.3	1.4	21.0	26.7	1.3
Nleu	48.6	62.1	1.3	22.5	27.3	1.2

TABLE IV

CAPACITY RATIO (k') OF AMINO ACIDS DERIVATIZED WITH OPA IN THE PRESENCE OF
MERCAPTOETHANOL

Mobile phase: 12.5% acetonitrile in 2.5 mM Cu(II)–L-proline

Amino acid	k' (L)
Tyr	17.3
Val	39.0
Nval	50.1
Try	127.3
Leu	121.9
Nleu	129.1

that primary amino compounds react stoichiometrically with OPA in alkaline me-
dium in the presence of a thiol. The thiol adds rapidly to OPA, forming an addition
product that is subsequently attacked by the primary amine. The reaction is fast,
goes to completion in less than 2 min, with virtually no detectable by-products: unlike
acylating reagents such as Dns chloride, OPA reacts with the amino group, not the
hydroxyl group of tyrosine. The product is highly fluorescent while the reagents are
not, thus offering excellent detection sensitivity. Post column derivatization with OPA
has become a favorite method for detecting amino acids emerging from HPLC ion-
exchange columns. A variety of thiols form adducts with OPA[18]. For detection of
amino acids after HPLC, the thiol generally used is mercaptoethanol. Precolumn
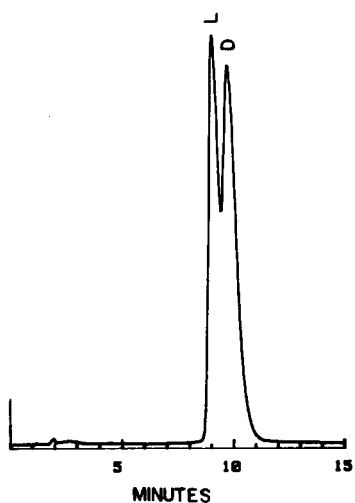derivatization with OPA and mercaptoethanol, followed by HPLC of the derivatives,

Fig. 4. Chiral separation of OPA derivative of normetanephrine. Mobile phase: 15% acetonitrile in a
buffer containing 5 mM L-proline, 2.5 mM CuSO$_4$ · 5H$_2$O and 2.0 g ammonium acetate, pH 7.0. Flow-
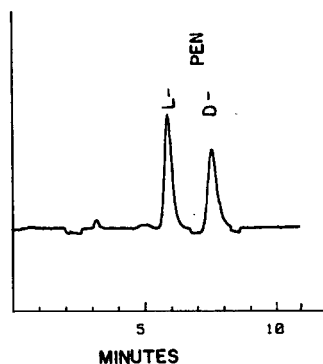rate 2.0 ml/min.

Fig. 5. Chiral separation of N-acetyl-D,L-penicillamine as the OPA derivatives. Mobile phase: 10% ace-
tonitrile in a buffer containing 5 mM L-proline, 2.5 mM CuSO$_4$ · 5H$_2$O and 2.0 g ammonium acetate, pH
7.0. Flow-rate 2.0 ml/min.

has also been used[16,17]. Aswad[19] substituted N-acetyl-L-cysteine for mercaptoethanol, thus transforming the OPA derivatives from enantiomers to diastereoisomers, in the hope of resolving enantiomers. With conventional HPLC on a reversed-phase column and a water–acetonitrile gradient, the only isomeric pair resolved was D,L-aspartic acid.

Examination of the molecular structure of the isoindole obtained by reacting OPA with N-acetyl-L-cysteine and an amino acid suggested that the carboxylate and the nitrogen of the N-acetylamino group of cysteine could form bi-dentate Cu(II) complexes. We therefore derivatized primary amino acids with OPA in the presence of N-acetyl-L-cysteine, injected them onto a reversed-phase column using Cu(II)–L-proline as the mobile phase and resolved the optical isomers (Figs. 1–3).

The separation of the OPA-amino acids generally follows those we observed previously with the Dns derivatives. The order of elution conforms to that of reversed-phase chromatography: the higher the carbon content, the bulkier the alkyl substituent on the α-carbon, the longer the retention (Table I). With isomers with equal numbers of carbons such as norleucine and leucine, the straight chain isomer was retained more, presumably because of stronger interaction with the stationary phase. The selectivity between the D- and L-pairs was also affected by the bulkiness of the alkyl substituents. The L-isomers of amino acids eluted before the D-isomers.

By substituting mercaptoethanol for NAC, the chiral separation disappeared (Table IV). This confirmed that the chiral separation achieved resulted from mixed chelate complexation at the carboxyl group and the N-acetylamino group of the N-acetyl-L-cysteine on the derivative rather than that of the analyte amino acid. We therefore tried to separate enantiomers of the derivative of normetanephrine, with OPA–NAC and achieved immediate success (Fig. 4).

Because of the dependence of the formation of the OPA adduct on the presence of a thiol group, we also studied the possibility of developing a procedure specific for enantiomers of sulfhydryl compounds. N-Acetylpenicillamine was chosen as an example of a sulfhydryl compound (N-acylation prevents reaction with the amine function). It was reacted with OPA in the presence of L-arginine as source of the chiral amine function and with no other source of sulfydryl agent. Clear separation of the D- and L-penicillamine derivatives was achieved with reversed-phase HPLC using a mobile phase containing Cu(II) and L-proline (Fig. 5).

CONCLUSION

The mixed chelation approach for resolving the optical isomers of Dns-amino acids was extended to resolve D- and L-isomers of free amino acids after pre-column derivatization with OPA in the presence of N-acetyl-L-cysteine. All OPA derivatives of primary amino acids were resolved. Study of the mechanism of complexation demonstrated that mixed chelation is independent of the chiral analytes. The optical isomers of amines and sulfhydryl compounds, which were not separated in our prior attempts with the dansyl derivatives, were successfully resolved as the OPA derivatives.

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