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Determination of amiprilose in human plasma by high-performance liquid chromatography with fluorimetric detection

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Abstract

A reversed-phase HPLC method to quantify amiprilose in human plasma is described. The method involves liquid–liquid extraction of amiprilose and the internal standard from plasma. The extracted compounds are derivatized with 1,8-naphthalic dicarboxylic acid using 2-chloro-1-methylpyridinium iodide as a coupling reagent. The derivatized products are separated on a reversed-phase column and monitored fluorimetrically using 280 nm and 340 nm as excitation and emission wavelengths, respectively. The derivatized products which exhibit two peaks on chromatogram, are shown to be the interconvertible isomers. This assay has been used in pharmacokinetic studies of amiprilose in humans.

Keywords: Amiprilose

1. Introduction

Amiprilose, *I*, an *O*-isopropylidene-3-*O*-3′-(*N*′,*N*′-dimethylamino-*n*-propyl)-D-glucofuranose, is claimed to have immunomodulatory and antiviral activities [1,2]. An analytical method has been reported which uses gas chromatography with electron capture detection after derivatization with heptafluorobutyric anhydride [1]. Recently, an abstract for the determination of *I* by a reversed-phase HPLC with amperometric detection was reported [3]. This paper reports an alternative method which utilizes chemical derivatization to form a fluorescent product to enhance the detectability. *I* is a substituted hexose and does not have a suitable absorptive chromophore for UV detection. There were several possible approaches to enhance the detectability: (a) acylation of the diol moiety to give a diester [4]; (b) periodate cleavage of the diol to generate formaldehyde, which is subsequently either trapped with a mixture of dimedone and ammonium acetate to form a fluorescent product [5], or reacted with acetylacetone and ammonium acetate to produce 3,5-diacetyl-1,4-dihydrolutidine, which is easily monitored by UV detection [6]; (c) acid hydrolysis of the acetonide moiety, and the resulting substituted glucose is separated on an ion-exchange column and trapped with either 2-cyanoacetamide in borate–phosphate buffer at 100°C [7,8] or ethylenediamine–borate buffer at 145°C [9]. Among these options, direct derivatization of diol would be the simplest approach. In the literature, various boronic acids have been used as a bifunctional reagent for the derivatization of diol, however, the products are not

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stable enough for reversed-phase LC analysis [10]. Among the UV tagging reagents for alcohols, *p*-nitrobenzoyl chloride, *p*-anisoyl chloride and 3,5-dinitrobenzoyl chloride have been used to analyze glycosides and carbohydrates [4,11–15]. 9-Anthroyl nitrile and 7-[(chlorocarbonyl)methoxy)]-4-methylcoumarin have been used as fluorotags for compounds containing hydroxy groups [16,17], but the reagents are not readily available. This paper describes the development of an HPLC–fluorescence detection method.

2. Materials and methods

2.1. Instrumentation

The liquid chromatography consisted of a Beckman Model 110A solvent delivery pump (Beckman Instruments, Berkeley, CA, USA) and Model 710B WISP (Waters Associates, Milford, MA, USA). The detector was a Shimadzu RF 530 fluorescence detector (ISI Instruspec, Walnut Creek, CA, USA). The integrator was a Hewlett-Packard Model 3390A (Hewlett-Packard, Santa Clara, CA, USA). A Hewlett-Packard 1040A photodiode array detector was used for on-line UV measurement. The heating block for the derivatization reaction was a Sybron Thermolyne 165001 dry bath (Fisher Scientific, Santa Clara, CA, USA). NMR spectrum was recorded on a Varian FT-80A spectrometer (Varian Associated, Walnut Creek, CA, USA). MS spectrum was recorded on a Finnegan GC–MS 4000 (Finnegan, Palo Alto, CA, USA) using low voltage mode and a DB-1 capillary column (15 m×0.032 mm).

2.2. Reagents and chemicals

I and the internal standard (*II*), an *O*-isopropylidene-3-*O*-3′-(*N*′,*N*′-diisopropylamino-*n*-ethyl)-D-glucofuranose, were obtained from Greenwich Pharmaceuticals (Fort Washington, PA, USA). 1,8-Naphthalic dicarboxylic acid was prepared from the hydrolysis of 1,8-naphthalic anhydride (Aldrich, Milwaukee, WI, USA) with 5% sodium hydroxide. 2-Chloro-1-methylpyridinium iodide (CMPI) was obtained from Fluka (Ronkonkoma, NY, USA). Ammonium acetate, HPLC grade acetonitrile and methanol were purchased from Fisher Scientific. Dry acetonitrile was prepared by drying over molecular sieve.

2.3. Preparation of plasma samples for standard curve or unknown and pre-column derivatization procedure

An aliquot of 0.25 ml of *I*-containing plasma and 12 μg of *II* in 100 μl of water were added to a screw-cap culture tube. The mixture was vortexed for 1 min. Then, 200 μl of 0.1 *M* sodium hydroxide and 6 ml of methylene chloride were added. The tube was capped and rotated on a Sepco tube rotator for 15 min. The mixture was centrifuged at 1500 *g* for 10 min and the lower-layer solution was transferred to a clean tube. The solvent was evaporated to dryness under nitrogen. Next, 2 mg of CMPI in 75 μl of acetonitrile and a mixture of 0.5 mg of 1,8-naphthalic dicarboxylic acid and 1.2 μl of triethylamine in 200 μl of acetonitrile were added to the dried residue. The mixture was then heated at 65°C in a heating block overnight. An aliquot of 20 μl was injected onto the HPLC column. The relative extraction recovery of *I* was determined by spiking internal standard into the extracted plasma and water samples. The peak height ratios were compared.

2.4. Chromatography

The separation of the derivatized products was achieved on a 250×4.6 mm Altex ultrasphere ODS column (5 μ) at ambient temperature. The mobile phase consisted of a mixture of methanol–1 *M* ammonium acetate–*N*,*N*–dimethyloctylamine–water (65:2.5:0.03:32.5, v/v/v/v). The flow–rate of mobile phase was 1 ml/min. The excitation and emission wavelengths of the detector were set at 280 and 340 nm, respectively.

2.5. Quantitation

The peak height ratios of *I* relative to *II* of the extracted unknown samples were evaluated against those of the spiked standards. The standard curve concentrations of plasma samples ranged from 0.103 to 8.24 μg/ml, and weighted linear regression analysis ($1/c^2$) was used to determine the slope,

intercept, and correlation coefficient. The concentration of *I* in the unknown samples was interpolated from the daily standard curve.

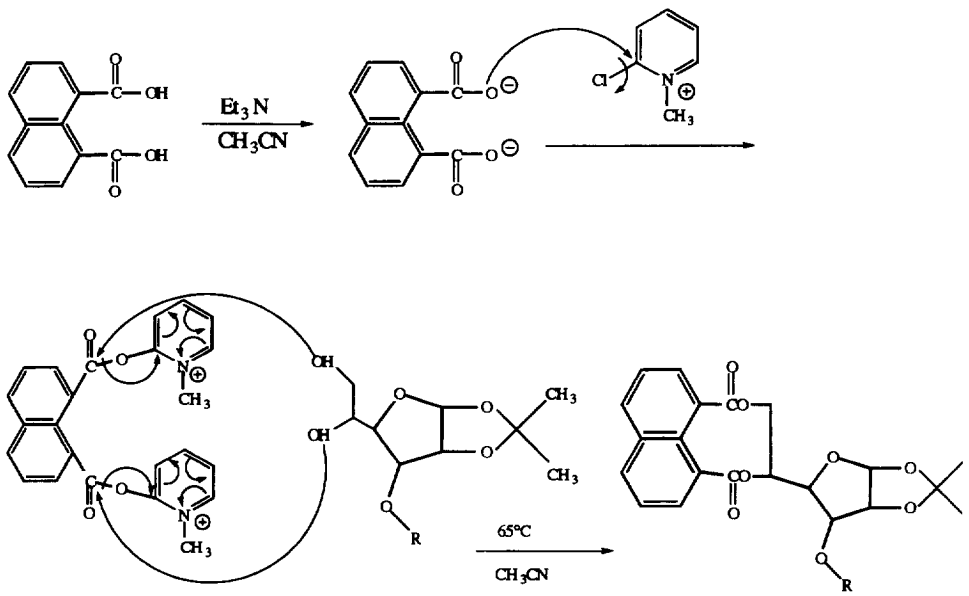
3. Results and discussion

3.1. Implementation of derivatization

In order to monitor the derivatization reaction, both silica gel TLC and reversed-phase HPLC techniques were used. For TLC analysis, both 2,4-dinitrophenylhydrazine (2,4-DNPH) and iodine vapor were used to visualize the reaction product. When *I* was treated with 2,4-DNPH, the specific spray reagent for aldehyde/ketone compounds, it showed the yellow color reaction due to the formation of acetone from the hydrolysis of the *O*-isopropylidene moiety. Iodine vapor was used to locate the unsaturated double bond (aromatic ring). In an attempt to prepare the diester of *I*, several substituted benzoyl chlorides were used. When *I* was mixed with *p*-anisoyl- or *p*-nitrobenzoyl chloride in pyridine at room temperature (RT), the expected ester was not formed. When the more reactive 3,5-dinitrobenzoyl chloride and the acylation catalyst *p*-dimethylaminopyridine (DMAP) were used concomitantly in the reaction, the reaction did proceed, but the products were found to be too labile to be analyzed by reversed-phase HPLC. However, when *I* was first treated with an excess of triethylamine in acetonitrile at RT, the subsequent addition of *p*-nitrobenzoyl chloride did produce some corresponding ester, but the ester also gradually decomposed in acetonitrile at RT. These results indicated that due to the instability issue, the formation of a benzoyl ester is not a feasible approach. Another approach using phenyl isocyanate [18] or naphthyl isocyanate to form a carbamate was also not successful. In spite of a recent report that glycerin was smoothly cleaved by periodic acid [19], the use of sodium iodate did not achieve the cleavage of the diol moiety of *I* under various conditions. Attempts to employ 1 *M* HCl to hydrolyze the acetonide moiety of *I* at 100°C via post-column reaction were also not successful. At this point, a new bifunctional reagent which could form a cyclic diester of *I* was explored. The seven-membered cyclic product was not prepared because the corresponding diacid needed for the reaction was not readily available. The eight- and nine-membered ring products could be in principle prepared from phthalic anhydride and 1,8-naphthalic anhydride, respectively. To conduct the esterification of diol, two different reaction conditions were examined: In the first method, dicyclohexylcarbodimide and DMAP were used as a coupling reagent and a catalyst [20], respectively. It was discovered that phthalic anhydride failed, but 1,8-naphthalic anhydride generated a product which showed positive color reaction toward 2,4-DNPH and I_2 vapor, indicating the product contained an *O*-isopropylidene and aromatic moieties. Nevertheless, the reaction yield was low. In the second method, CMPI was used as a coupling reagent [21,22], and both anhydrides produced a better yield than the first method. However, 1,8-naphthalic anhydride gave a higher yield than that of phthalic anhydride. These results illustrated that it is probably easier to form a cyclononane product with 1,8-naphthalic anhydride than a cyclooctane product with phthalic anhydride. It was also observed that 1,8-naphthalic dicarboxylic acid, the product formed from the hydrolysis of the corresponding anhydride, showed better solubility in the reaction medium than the anhydride, therefore, 1,8-naphthalic dicarboxylic acid and CMPI were used in the subsequent derivatization reaction (Fig. 1). After a number of trials, it was found that the derivatization yield reached a maximum after heating the reaction mixture at 65°C for 12 h, therefore, the reaction was performed at 65°C in a heating block overnight.

3.2. Characterization of the derivatization product

The derivatization products of *I* were separated on an ODS column under isocratic conditions. Fig. 2 illustrates a typical chromatogram showing two peaks of the derivatives which eluted at 18 and 21 min, respectively. The second peak with a longer retention time was the major product. The peak height ratio of these two peaks was approximately 1:3 and remained reasonably constant under the present LC conditions. A similar phenomenon also occurred with the internal standard. It was therefore, of interest to explore why the ratio of these two peaks was constant. Attempts to isolate these two



I. Amiprilose: $R=C_3H_6N(CH_3)_2$

II. Internal Standard: $R=C_2H_4N(i\text{-}C_3H_7)_2$

Fig. 1. Chemical derivatization of amiprilose (*I*) and internal standard (*II*) with 1,8-naphthalic dicarboxylic acid.

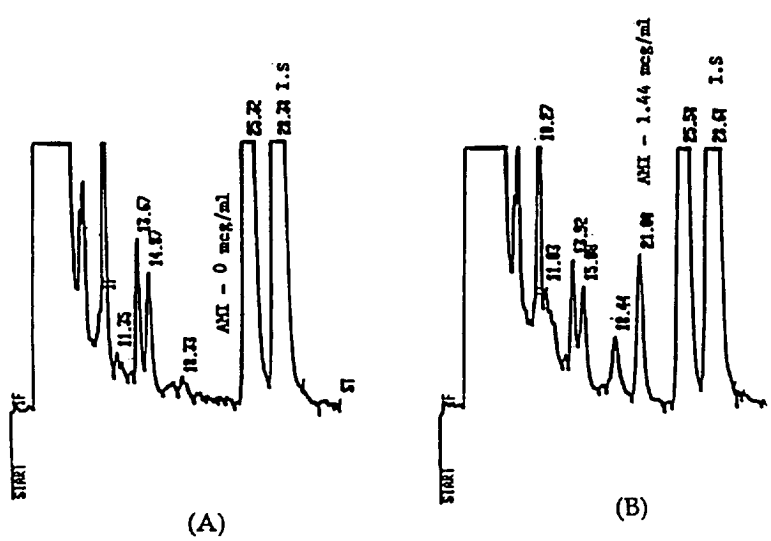
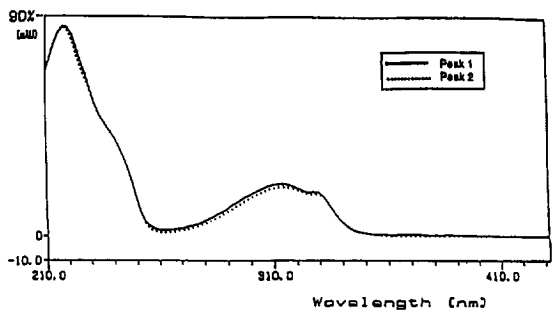


Fig. 2. Typical chromatograms of (A) blank plasma spiked with *II*, (B) blank plasma spiked with *II* and 1.44 μg/ml of *I*.



	Peak 1	Peak 2
Retention Time (min)	25.08	28.47
Attenuation Adjustment (mAu)	89.4	291.2
Absorbance at 218 nm (mAu)	76.7	249.6

Fig. 3. On-line HPLC–UV spectra of amiprilose derivatives after attenuation adjustment.

components both by silica gel TLC and reversed-phase HPLC revealed that the subsequent HPLC analysis of each isolated fraction still showed two peaks, and these two peaks gradually interconverted, implying that something was going on from the time of collection to the time of analysis of each fraction. An on-line UV photo-diode array detector was then used to measure the UV spectrum of each peak during HPLC separation. Both peaks showed absorption maxima at 218, 313, and 320 nm. With a proper adjustment of the attenuation of the instrument, the two UV spectra superimposed completely, indicating that these two peaks might have identical chromophores (Fig. 3). In addition, no other impurities could be found underneath the peaks when different wavelengths were used for measurement [23]. The proton NMR spectrum of the derivatized product was not completely assigned in the aliphatic regions, but the presence of aromatic protons ($CDCl_3$ δ: 7.6–8,6, *m*, 6H) indicated the presence of a naphthalic ring. The mass spectral data showed a quasi-molecular ion

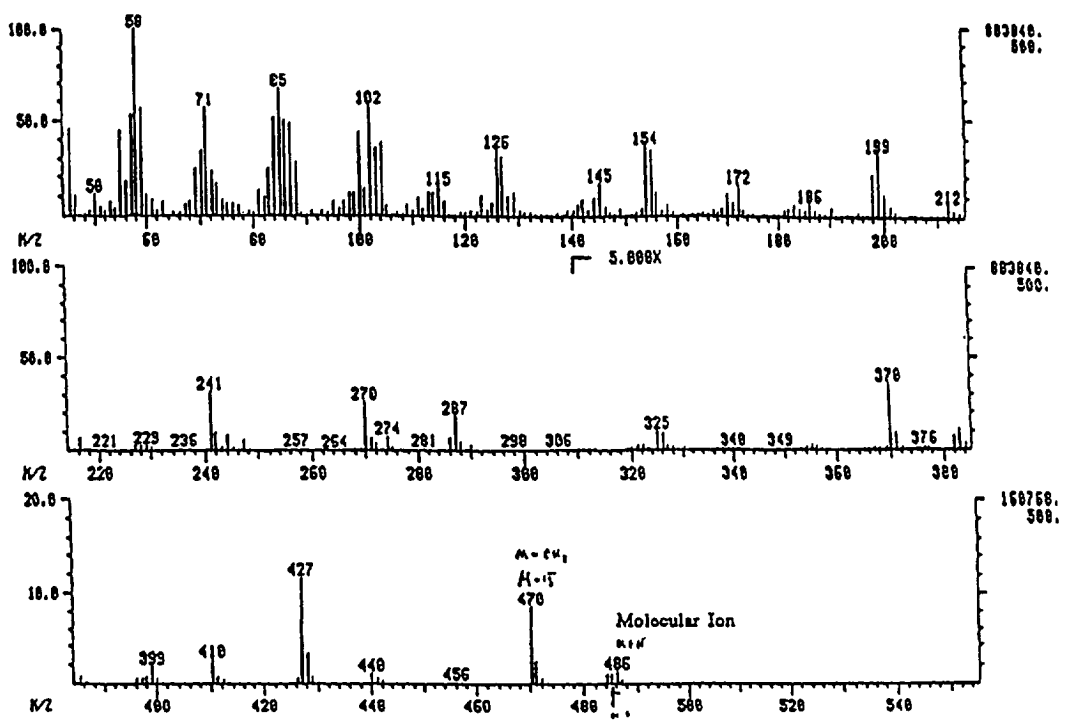


Fig. 4. Mass spectrum of amiprilose derivative.

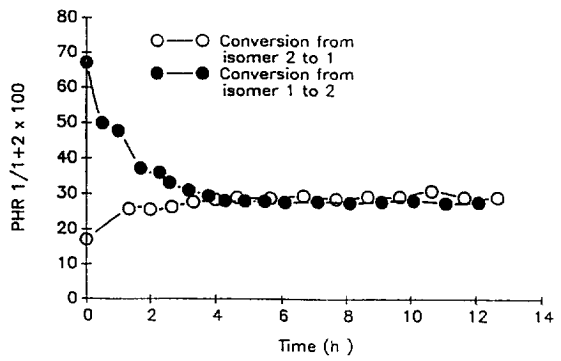


Fig. 5. Interconversion of amiprilose derivatives. The derivative with the shorter retention time is designated as isomer 1.

at 486 (M+H) which is in agreement with the proposed cyclic structure with a molecular weight of 485 (Fig. 4). Others were *m/z* 470 ($M-CH_3$), *m/z* 427 ($M-CH_2N(CH_3)_2$) and *m/z* 126 for the naphthalic ring.

3.3. Chromatographic interconversion experiment

By collecting a fraction corresponding to each of the derivatized products from the HPLC column, and reinjecting aliquots of each of these fractions onto the HPLC column within 5 min after the collection and at intervals over more than 12 h, an interconversion rate was measured. Fig. 5 shows that the interconversion occurred readily and the two products reached to equilibrium in approximately 5 h at RT. The ratio of these two peaks was 1:3 and remained constant. The half-life of this conversion from isomer 1 to 2 and from isomer 2 to 1 was approximately 0.9 h and 1 h, respectively. In this study, no attempt was made to assign the conformation of each conformer.

Table 2
Inter-day precision data for *I* in plasma

Spiked concentration (μg/ml)	Found concentration (mean±S.D., $n=6$) (μg/ml)	C.V. (%)
0.185	0.193±0.002	1.22
0.824	0.829±0.035	4.24
2.060	2.047±0.093	4.56
5.150	5.201±0.169	3.25

3.4. Specificity, linearity, intra- and inter-day precision

Typical chromatograms obtained from control plasma spiked with *II*, and plasma spiked with *I* and *II* are shown in Fig. 2. Both *I* and *II* derivatives appeared as two peaks, and the second peak of each pair was used for quantitation. The retention times of the second peak for the derivatives of *I* and *II* were column dependent, and were approximately 21 and 28 min for a typical run. No interfering peaks were observed. Since at the time of analysis after derivatization, the peak height ratio of peak 1 versus 2 remained constant, the reaction was used as a quantitative analysis. The typical calibration function of *I* is $y=0.04847x-0.0011$, $r^2=0.9988$. For assay validation, five sets of plasma samples at four concentrations were analyzed on the same day or on five different days to obtain intra- and inter-day precision data, respectively. The results are summarized in Tables 1 and 2. The coefficients of variation were less than 10%. The intra-day accuracy was greater than 90%. The frozen stability of *I* in plasma at −20°C was demonstrated by examining the frozen

Table 1
Intra-day precision and accuracy data for *I* in plasma

Spiked concentration (μg/ml)	Found concentration (mean±S.D., $n=6$) (μg/ml)	C.V. (%)	% difference[a]
0.185	0.191±0.012	6.29	3.12
0.824	0.814±0.059	7.30	6.49
2.060	2.033±0.070	3.44	2.86
5.150	5.064±0.204	4.03	3.65

[a] % difference=mean of absolute % difference.

Table 3
Frozen stability for *I* in plasma

Spiked concentration (μg/ml)	Found concentration (mean±S.D., $n=31$) (μg/ml)	C.V. (%)
0.258	0.285±0.041	14.8
0.770	0.750±0.055	7.35
1.53	1.49±0.129	8.63
3.01	3.07±0.185	6.01

n=number of analyses conducted over a one-month period.

QC samples which were analyzed along with clinical samples. The results are shown in Table 3. Amiprilose was stable for at least over a one-month study period.

3.5. Recovery

The recovery of *I* in the plasma sample was found to be better than 90%.

3.6. Determination of amiprilose in human plasma

The present assay has been used to analyze human plasma samples for pharmacokinetic studies. A typical plasma concentration versus time profile of one volunteer taking two capsules with and without a standard breakfast are shown in Fig. 6. Thus, this assay is suitable for measuring the concentration of amiprilose in plasma.

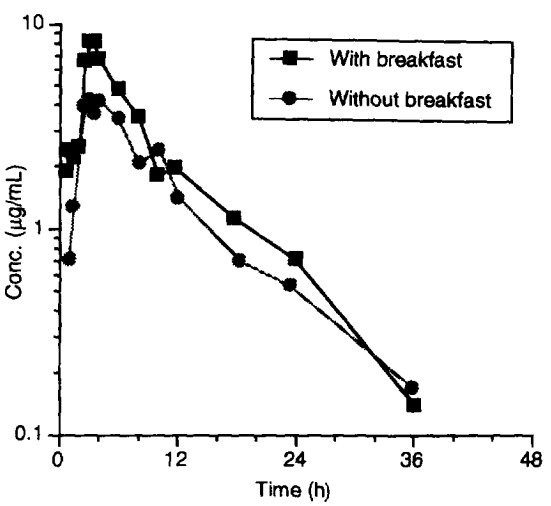


Fig. 6. Plasma concentration of amiprilose versus time in one volunteer taking two capsules formulation (2×500 mg) with or without a standard breakfast.

4. Conclusions

The present assay which utilizes naphthalic 1,8-dicarboxylic acid as a bifunctional fluorotag to react with the diol functional group of *I*. The derivatization reaction generated two interconvertible isomers which were resolved on a reversed HPLC column. The derivatized product successfully enhanced the detection sensitivity and provided adequate detectability for therapeutic monitoring. The present methodology might be extended to analyze other compounds which contain diol, diamine or amino-alcohol functional groups whenever a suitable chromophore is needed to enhance the detectability and the chemistry of the reaction is feasible.

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