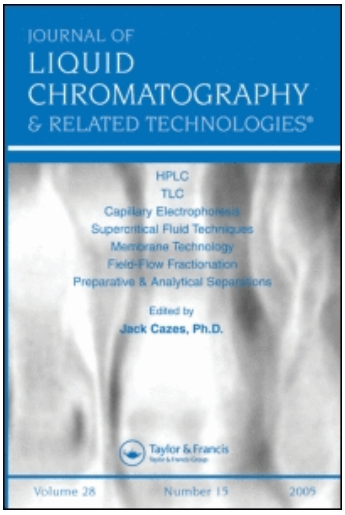

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QUANTIFICATION OF ^{14}C-LABELED AMINO ACIDS BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

This study provided the first evidence indicating that o-phthalaldehyde-amino acid-derivatives are unstable during high performance liquid chromatographic separation on a reverse-phase C^{18} column. Instability of these derivatives on the column resulted in the formation of ^{14}C-labeled degradation products, and caused excessive ^{14}C-fronting of amino acid peaks. The column halflives of glutamate, arginine, and ornithine were 16, 40, and 54 minutes, respectively. Derivative break-down during derivatization also gave rise to these degradation products in the ^{14}C-distribution profile. Thus, if OPA-derivatization is used to quantify the ^{14}C-distribution in an unknown mixture of amino acids it is necessary to determine correction factors for each amino acid of interest. A method which uses stable phenylthiocarbamate-derivatives did not exhibit these problems and is therefore more suitable for the quantification of ^{14}C-amino acids.

INTRODUCTION

The reaction between o-phthalaldehyde (OPA), 2-mercapto-ethanol (MCE) and amino acids produces fluorescent, hydrophobic

derivatives (1). This chemistry is extensively utilized in the separation of amino acids by reverse-phase high performance liquid chromatography (2-4). One inherent drawback of this chemistry is the instability of OPA-derivatives (5), but with the advent of automated sampling and mixing systems the fluorescence monitoring of these derivatives is no longer a problem.

The effect of OPA-derivative instability on the quantification of ^{14}C-labeled amino acids has not been documented. A potential problem exists because of a difference in discrimination between fluorescence and radioactivity detection. As OPA-derivatives degrade a non-fluorescent product, which will not produce additional peaks during fluorescence monitoring, is formed (5). However, the breakdown products from OPA-derivatives of ^{14}C-amino acids will also be ^{14}C-labeled, and therefore might produce additional peaks when ^{14}C-distribution among amino acids is monitored. This problem has been ignored by previous workers separating ^{14}C- and ^{15}N-labeled amino acids after OPA-derivatization (4,6).

In this study the effect of OPA-derivative instability on ^{14}C-amino acid quantification was investigated using ^{14}C-labeled glutamate (Glu), arginine (Arg) and ornithine (Orn), compounds which collectively possess retention times encompassing an entire chromatographic run. In particular, arginine and ornithine derivatives were compared for their degradation characteristics in the derivatization solution, in one of the HPLC solvents, and on the column; these OPA-amino acids are known to possess

contrasting stability characteristics (7). This study was facilitated by comparing the OPA-derivative procedure with another reverse-phase HPLC procedure which utilizes stable phenylthiocarbamate (PTC)-amino acid derivatives, a method which has recently become popular (8-9).

EXPERIMENTAL

Materials and Reagents

The solvents used for liquid chromatography were HPLC grade, and all other chemicals, including unlabeled amino acids, were analytical-reagent grade. L-[1-^{14}C]glutamate, L-[1-^{14}C]ornithine, and L-[U-^{14}C]arginine were purchased from New England Nuclear. Standards were prepared using a Pierce Amino Acid Calibration Standard, together with unlabeled citrulline, glutamine, asparagine, 4-aminobutyric acid and argininosuccinic acid. Unlabeled standards containing only arginine or ornithine were also prepared.

The PTC-derivatizing reagent contained PTC/methanol/water/triethylamine, 7/1/1/1 by volume, and the OPA-derivatizing reagent was prepared daily by adding 1.1 mg OPA and 40 ul MCE to 10 ml 0.4 M NaBorate (pH 10.4)/methanol, 9/1 by volume.

Deionized-distilled water was used for all PTC-derivative procedures, and deionized water purified by a Millipore Milli-Q^{TM} Water System was used for the OPA method. Solvents were filtered through 0.45 uM Millipore filters and degassed under vacuum before use.

HPLC Procedure for PTC-Derivatives

The HPLC system consisted of two Waters 6000A pumps, a Waters 440 Fixed Wavelength Absorbance Detector set a 254 nm, a Waters Temperature Control Module set at 46^{0}C, a Waters 710B WISP^{TM} Sample Processor and a Waters 840 Data and Chromatography Control Station. The column was a Waters Pico-Tag^{TM} column for physiological fluids (3.9 mm X 30 cm). Fractions were collected using a Cygnet Isco Fraction Collector and radioactivity was quantified using a Wallac 1212 RackBeta Scintillation Counter.

Twenty five microlitres of unlabeled standard (1.25 mM/amino acid), together with 1.5 X 10^4 Bq of one ^{14}C-amino acid (Glu, Arg or Orn), was vacuum dried, resuspended in 50 ul methanol/water/ triethylamine, 2/2/1 by volume, and redried under vacuum. Fifty microlitres of derivatization solution was then added per sample, and the mixture sat for 35 minutes before being vacuum dried and resuspended in 600 ul of 5 mM Na_2HPO_4 (pH 7.4)/ acetonitrile, 95/5 by volume. Ten microlitres of sample (500 pmoles/amino acid) was injected per run.

Solvent A consisted of 70 mM NaAcetate (pH 6.55)/ acetonitrile, 97.5/2.5 by volume, and Solvent B contained acetonitrile/methanol/distilled water, 45/15/40 by volume. Gradient P-1 was as follows (flow rate=1.0 ml/min): 0% B, 13.5 min; 3-5% B, 10.5 min, curve 8; 5-9% B, 6 min, curve 5; 9-34% B, 20 min, curve 6; 34% B, 12 min; 34-100% B, 3 min, curve 6; 100% B, 10 min. The system was equilibrated for 7 minutes using 0% B. The eluate was collected at 48 second intervals, 5 ml of Amersham

ASC II Scintillation Cocktail was added per fraction, and the vials were counted for 2 minutes.

HPLC Procedure for OPA-Derivatives

The HPLC system included two Waters M-45 pumps, a Waters 420 Fluorescence Detector set a 360 nm excitation and 455 nm emission, a Waters Temperature Control Module set at 44^{0}C, a Waters U6K Universal Liquid Chromatography Injector, a Waters 680 Automated Gradient Controller, and a Waters Resolve^{TM} C^{18} Guard-Pack Precolumn Insert. A Waters 5 um C^{18} Resolve^{TM} column (3.9 mm X 15 cm) was used. Radioactivity was quantified using a Ramona-D (Raytest Instruments, Quebec, Canada) flow through isotope detector containing a 500 ul yttrium silicate solid cell. The eluate was collected using an LKB Bromma 2111 Multirac Fraction Collector, and the radioactivity quantified using a Beckman LS 6800 Scintillation Counter.

Fifty microlitres of unlabeled standard (12.5 uM/amino acid), together with 308 Bq of one ^{14}C-amino acid was added to 262 ul of derivatizing reagent; this was allowed to react for 2 minutes, and then 250 ul of derivatized sample (500 pmoles/amino acid) was manually injected.

Solvent C consisted of 50 mM NaAcetate + 50 mM Na_2HPO_4 (pH 7.4)/methanol/tetrahydrofuran, 96/2/2 by volume, and solvent D contained methanol/water, 62/38 by volume. Gradient 0-1 was as follows: 10-35% D, 5 min, curve 7; 35-80% D, 14.5 min, curve 7; 80-100% D, 4 min, curve 6; 100% D, 3 min. Gradient 0-2 was as

follows: 10-16% D, 3.5 min, curve 9; 16-19% D, 12 min, curve 6; 19-62% D, 7 min, curve 10; 62-100% D, 8 min, curve 6; 100% D, 3 min. Gradient 0-3 was: 10-11% D, 19.5 min, curve 9; 11-16% D, 19 min, curve 10; 16-62% D, 7 min, curve 5; 62-100% D, 8 min, curve 5; 100% D, 3 min. The equilibration time for the gradients was 7 minutes at 10% D and all flow rates were 1.5 ml/min. The eluate was collected at 24 second intervals, 3.4 ml of Beckman Ready-Solv HP/b scintillation cocktail was added per fraction, and the vials were counted for 2 minutes.

Statistical Analysis

Each value is the mean of duplicate injections from a single preparation repeatedly injected at times selected randomly. Treatment of the data was conducted using exponential regression analysis (10) where indicated. Levels of significance of 5% and 10% are indicated by ** and *, respectively.

RESULTS

Quenching and Luminescence Characteristics

Chemical quenching was not observed using either the PTC- or OPA-methodologies, in conjunction with fraction collection and quantification of ^{14}C by liquid scintillation spectrometry (data not shown); this agrees with Early and Ball (9), who found no chemical quenching using the PTC-methodology. This is also supported by the identical ^{14}C-distribution patterns obtained with

the isotope detector and fraction collection-liquid scintillation spectrometry procedures (no corrections for quenching performed) for OPA-derivatives (Fig. 1-3), since yttrium silicate solid cells are free of chemical quenching effects (11).

The radioactivity quantified in the OPA- and PTC-derivatived samples was identical to underivatized samples not separated by HPLC (data not shown), indicating that photoluminescence and chemiluminescence was not occurring at wavelengths equal to the photomultiplier tube sensitivity spectrum. In addition, no ^{14}C-contamination by preceding runs was observed for either methodology.

Separation Profiles

The ^{14}C-distribution patterns for glutamate are shown in Fig. 1. With the OPA-derivative procedure 5% of total ^{14}C was recovered in the void, 76% in the glutamate peak and 19% in residual ^{14}C. The residual ^{14}C was not in a distinct peak but was distributed between the void and glutamate peak. A different pattern was observed for the PTC-derivative procedure; 3% of total ^{14}C was in the void, 90% in the glutamate peak and 7% in an unknown peak.

With the OPA-arginine derivative (Fig.2) 7% of total ^{14}C was recovered in the void, 2% in peak 1, 61% in the arginine peak and 30% was residual ^{14}C. The residual ^{14}C was distributed between peak 1 and the arginine peak. In contrast, the recovery of ^{14}C in the arginine peak was improved to 93% using the PTC-methodology.

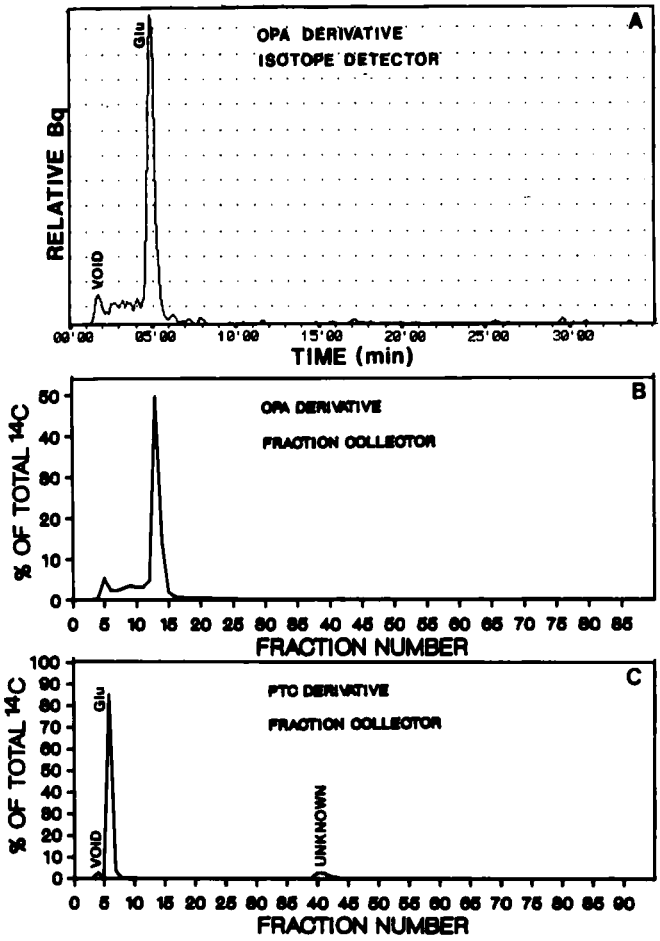

FIGURE 1. HPLC analysis of OPA- and PTC-derivatives of ^{14}C-l-glutamate. The chromatograms illustrate the distribution of ^{14}C in the eluate: from the OPA-derivatized sample, using gradient O-2, detected either by isotope detection (A) or fraction collection-liquid scintillation spectrometry (B); and from the PTC-derivatized sample, using gradient P-1, detected by fraction collection-liquid scintillation spectrometry (C). All data is background corrected.

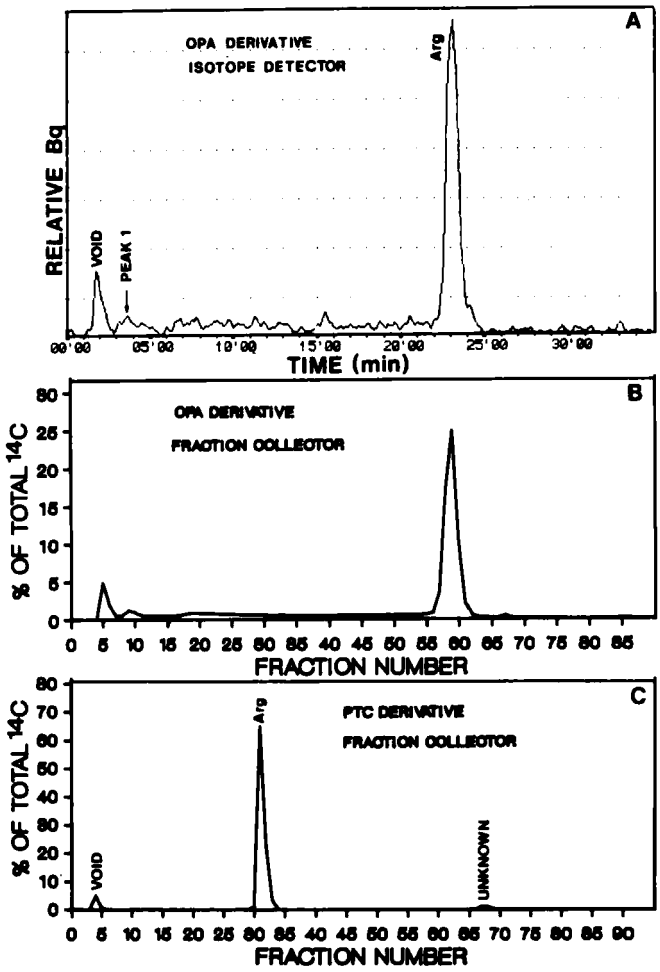

FIGURE 2. HPLC analysis of OPA- and PTC-derivatives of ^{14}C-U-arginine. See Fig. 1 for a further description of methods.

The ^{14}C-distribution pattern observed for the OPA-ornithine derivative (Fig. 3) was the most complicated: peak 1, 5% of total ^{14}C; peak 2, 2%; peak 3, 6%; peak 4, 14%; ornithine peak, 65%; and residual, 8%. Only two peaks were observed with the PTC-ornithine derivative; 96% in the ornithine peak and 4% in a reagent peak. Thus, the high number of peaks in the OPA-ornithine samples could not be attributed to amino acid impurities.

Degradation Characteristics of OPA-Derivatives

The data in Fig. 4A demonstrate that the OPA-arginine-derivative degraded from 2 to 60 minutes in OPA+MCE, with a concomitant increase in peak 1 (non-fluorescent) from 5% to 23% of total ^{14}C. OPA-ornithine also degraded from 1 to 30 minutes in OPA+MCE (Fig. 4B), with a concomitant increase in peak 4 (non-fluorescent) from 11% to 38% of total ^{14}C, in peak 5 (non-fluorescent) from 0% to 3%, and in residual ^{14}C from 5% to 16%. The total loss of ^{14}C-arginine and ^{14}C-ornithine up to 60 and 30 minutes, respectively, in solvent C (40 ul of derivatized sample mixed with 400 ul of solvent) was not different from the loss in OPA+MCE (data not shown).

As retention times on the column were increased there was a decrease in ^{14}C in the arginine peak, and a concomitant increase in residual ^{14}C (Fig. 5A). The ^{14}C in the ornithine peak also decreased as retention times were increased, and residual ^{14}C increased (Fig. 5B).

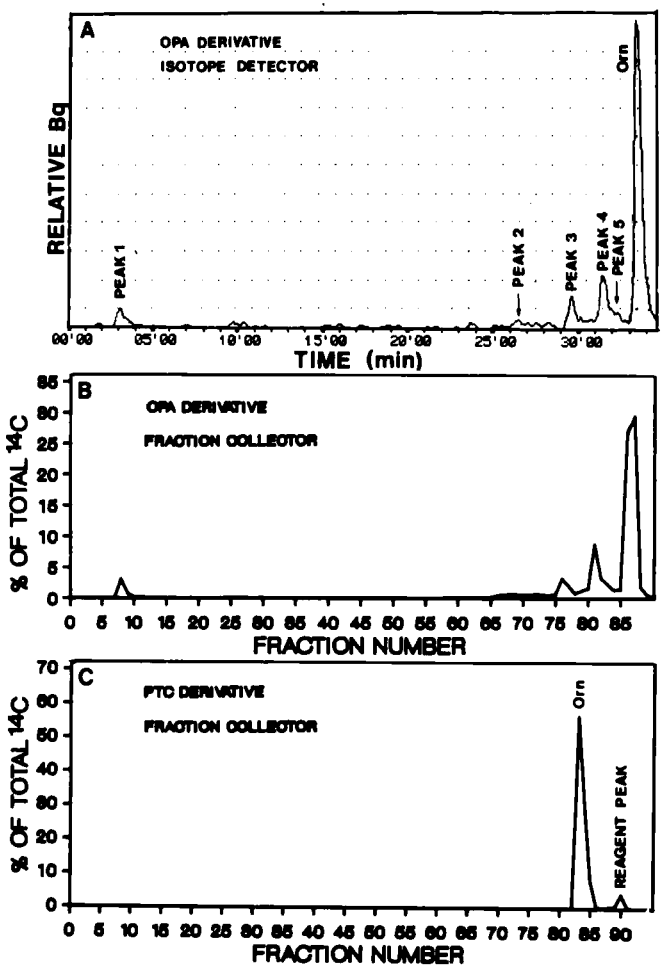

FIGURE 3. HPLC analysis of OPA- and PTC-derivatives of ^{14}C-l-ornithine. See Fig. 1 for a further description of methods.

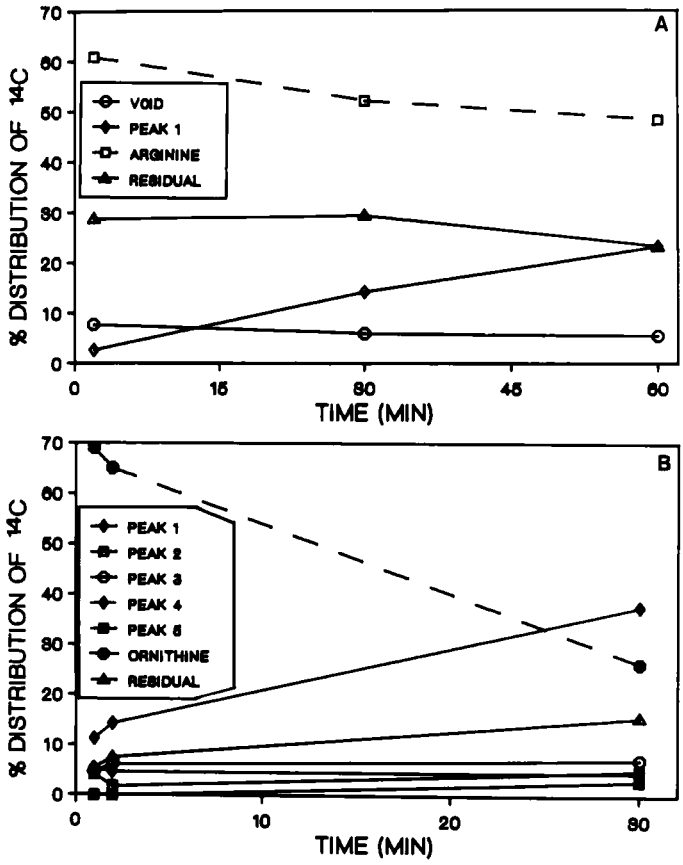

FIGURE 4. The stability of OPA-derivatives of ^{14}C-U-arginine (A) and ^{14}C-l-ornithine (B) in OPA+MCE. The retention times of the plotted peaks are indicated in Fig. 2 (arginine) and Fig. 3 (ornithine), and any ^{14}C not in these peaks is considered residual ^{14}C.

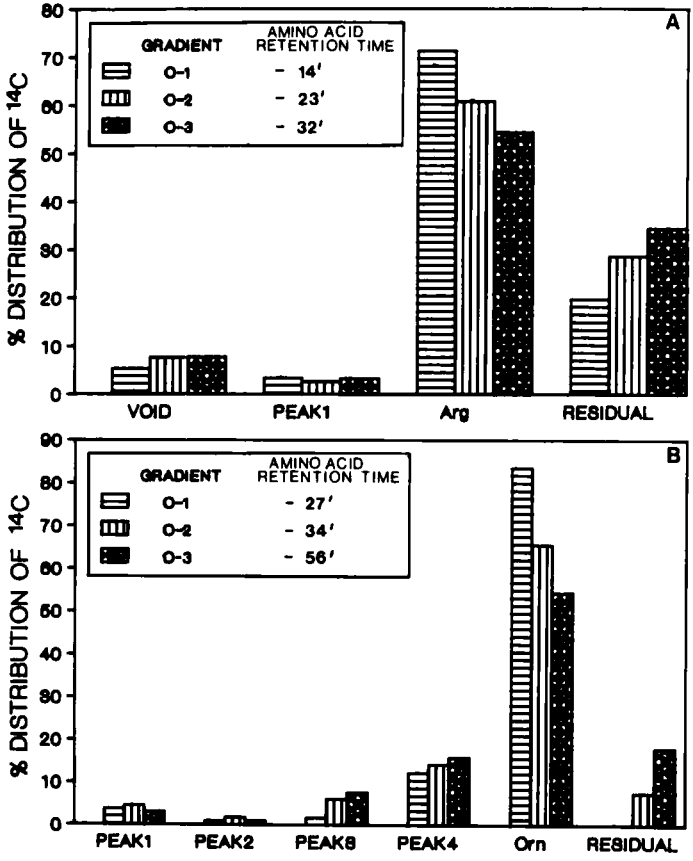

FIGURE 5. The stability of OPA-derivatives of ^{14}C-U-arginine (A) and ^{14}C-l-ornithine (B) on a Waters Resolve^{TM} C^{18} reverse-phase column. Retention times of peaks using gradient O-2 are shown in Fig. 2 (arginine) and Fig. 3 (ornithine). Retention times of peaks using gradient O-1 were: (1) Arginine- void (2'01"), peak 1 (4'09"), Arg (13'53"); (2) Ornithine- peak 1 (3'09"), peak 2 (20'30"), peak 3 (23'37"), peak 4 (25'29"), Orn (27'22"). Retention times of peaks using gradient O-3 were: (1) Arginine- void (2'01"), peak 1 (3'50"), Arg (32'04"); (2) Ornithine- peak 1 (3'18"), peak 2 (49'33"), peak 3 (51'34"), peak 4 (53'26"), Orn (55'56").

Halflives of OPA-Derivatives

The halflives of the OPA-derivatives in OPA+MCE and on a Waters Resolve^{TM} C_{18} reverse-phase column are shown in Fig. 6. The OPA+Column curves represent the experimentally determined loss of ^{14}C-amino acids; the losses were due to a combination of instability in OPA+MCE and on the column. Column curves represent the loss of ^{14}C-amino acids as a result of instability on the column; corrections were made to compensate for OPA-derivative losses in OPA+MCE during the 2 minute derivatization time. The OPA curves represent the loss of ^{14}C-amino acids in OPA+MCE with time, prior to injection on the column, derived mathematically as follows: % of total radioactivity= (OPA+Column%)/$e^{-(0.693/T_{\frac{1}{2}}}$ (column)) X retention time (gradient 0-2).

Halflives were calculated using both ^{14}C-distribution and fluorescence data; they showed the same trends with the exception of the halflife for ornithine on the column. The halflives in OPA+MCE for arginine and ornithine (average of the ^{14}C and fluorescent data) were 156 minutes and 22 minutes, respectively; the average column halflives for arginine and ornithine were 40 minutes and 54 minutes, respectively. Stobaugh et al. (12) have also shown that OPA-derivatives exhibit first-order degradation kinetics. The difference between the OPA+Column curves and OPA curves (Fig. 6A) represents the break-down of OPA-derivatives while on the column. With this information column losses using gradient 0-2 were estimated as 35% (arginine) and 37% (ornithine) of the OPA-derivative originally injected onto the column.

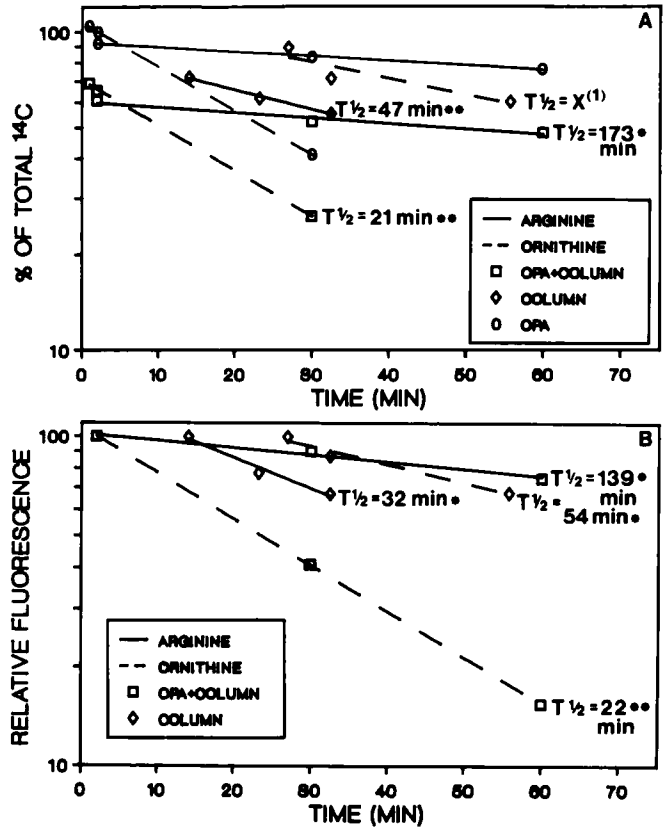

FIGURE 6. Halflives of OPA-derivatives of arginine and ornithine in OPA+MCE and on a Waters Resolve^{TM} C^{18} reverse-phase column. Halflife values were determined using ^{14}C-distribution data (A) and fluorescence data (B). See RESULTS-Halflives of OPA-Derivatives for a description of the curves. All data were fitted to an exponential regression, and halflives were determined from the regression equations. (1) No significant exponential relationship.

DISCUSSION

The instability of OPA-derivatives has previously been demonstrated using fluorescence data (7, 13-14), but the approach used in those studies did not allow monitoring of breakdown products since they do not fluoresce. However, the approach used in the present study allowed the determination of the distribution of OPA-amino acid derivatives and breakdown products after separation by reverse-phase HPLC. Arginine and ornithine degradation in OPA+MCE was accompanied by an increase in peak 1 (Fig. 4A) and peak 4 (Fig. 4B), respectively. These non-fluorescent peaks possibly correspond to the compound 2,3-dihydro-1H-isoindole-1-one, which is the final product of OPA-derivative break-down (12). The percentage of total label present as residual ^{14}C was considerable for all of the ^{14}C-amino acids tested. These observations could not be explained by ^{14}C-contamination, photoluminescence and/or chemiluminescence. The possibility of a column surface effect resulting in excessive fronting could not be the explanation since there was not a parallel fluorescence response. The ^{14}C-distribution patterns suggest that the residual ^{14}C was an OPA-degradation product formed on the column. For example, continuous arginine degradation on the column would result in the formation of a dihydro-1H-isoindole, which would be eluted after peak 1; the process would continue until arginine eluted, producing ^{14}C-fronting as was observed.

Cooper et al.. (15) investigated the column stability of OPA-derivatives by stopping solvent flow for increasing lengths of

time after samples reached the column. They observed a gradual decline in fluorescence for all of the amino acids tested. This approach was informative but it did not demonstrate the actual degradation rate occurring during a chromatographic run. In this study column degradation rates were determined for a flowing system by changing amino acid retention times (Fig. 5 and 6). Assuming the residual ^{14}C is a column-derived degradation product, the OPA-arginine derivative ^{14}C% at time 0 in the chromatographic run would equal residual ^{14}C%+Arg ^{14}C%. Using the equation $A=A_0e^{-kt}$, where A=Arg ^{14}C%, A_0=Arg ^{14}C%+residual ^{14}C% and t=retention time, and the OPA+MCE instability samples from 2-60 minutes (Fig. 4A), the average column halflife of arginine was calculated as 40 minutes. This halflife value corresponds to the halflife determined in Fig. 6. The column halflife of glutamate was calculated as 16 minutes using the equation described here.

In the present study the halflives of arginine and ornithine in OPA+MCE (Fig. 6) were respectively 4 and 11 times longer than those determined by Turnell and Cooper (7). The previous study used an OPA concentration 3750 times the amino acid concentration, whereas this study only used a 15 times excess. It is known that degradation rates increase as the OPA concentration increases, possibly through an OPA catalytic effect (12); this might provide an explanation for the discrepancy in halflives between the two studies.

The instability of OPA-derivatives in OPA+MCE and on the column has practical implications for the quantification of a

mixture of labeled amino acids. To correctly quantify labeled OPA-amino acids correction factors have to be determined to compensate for the differential loss of label. For example, in the case of arginine, ornithine, and glutamate the correction factors, determined using gradient 0-2, were 1.56, 1.54, and 1.22, respectively. Radioactivity also may have to be subtracted from amino acid peaks to remove contributions by the labeled OPA-degradation products of other amino acids. This correction would be most difficult when label is present in many amino acids. Since all of the label in an OPA-amino acid derivative did not elute in discrete peaks there is the potential for peak masking. This problem would be most significant when some amino acids are highly labeled relative to others and when an amino acid, such as ornithine (Fig. 3), has a complicated labeling distribution pattern. In addition, peaks in the ^{14}C-profile resulting from the degradation products of an OPA-amino acid, as seen for ornithine, could be mistaken for other ^{14}C-amino acids.

The present study showed that the quantification of labeled amino acids by reverse-phase HPLC using OPA-derivatives has inherent problems and it would be more suitable to use a system which uses stable derivatives such as PTC-amino acid derivatives. However, if such a system is not available, and it is necessary to use OPA-derivatives for the quantification of labeled amino acids as some researchers have done (4,6), corrections to the amino acid label profiles will be required.

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