Journal of Chromatography, 164 (1979) 417—426
Biomedical Applications

CHROMBIO. 417

NEW PROCEDURE FOR ISOLATION OF AMINO ACIDS BASED ON SELECTIVE HYDROLYSIS OF TRIMETHYLSILYL DERIVATIVES

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(Received May 25th, 1979)

SUMMARY

A rapid procedure for the isolation of amino acids from physiological fluids by class separation suitable for gas chromatographic and gas chromatographic—mass spectrometric analysis is described. A physiological fluid such as plasma is adjusted to pH 2 and extracted with diethyl ether to remove organic acids and neutrals. After precipitation of proteins with trichloroacetic acid, the aqueous plasma is dried and derivatized by trimethylsilylation. Organic compounds like sugars and amino acids are rendered soluble in petroleum ether leaving inorganic salts when the soluble layer is transferred. Separation of sugars from amino acids is achieved by taking advantage of the different rates of aqueous hydrolysis of the trimethylsilyl (TMS) derivatives. Mixing the petroleum ether extract with a small volume of water results in two phases. The petroleum ether layer contains TMS-sugar constituents of plasma and the aqueous layer contains free amino acids and amines. This procedure was used to isolate L-dopa, 3-O-methyldopa and tyrosine from human plasma in a quantitation assay using ^{18}O-labelled amino acids and gas chromatography—mass spectrometry.

INTRODUCTION

There has been a multitude of methods developed for the separation and analysis of amino acids present in physiological fluids involving ion-exchange, thin-layer, high-performance liquid, paper, and gas chromatographic techniques. Because of some unique advantages, amino acid analysis by gas chromatography has become a widely used procedure and has been recently reviewed [1]. The gas chromatographic (GC) procedures offer advantages of speed, sensitivity, and applicability to most naturally occurring amino acids and complete resolution of the twenty protein amino acids can be achieved with a single column in a total chromatographic time of less than one hour [2]. The necessity of making a derivative of the amino acid to increase thermal stability and to improve chromatographic behavior is often

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considered to be a disadvantage of the GC techniques; however one can use this to advantage. For example, derivatives suitable for electron-capture GC detection can be made, which profoundly increase the analytical sensitivity of amino acid quantitation [3]. Recent advances in combined gas chromatography—mass spectrometry (GC—MS) have made GC increasingly attractive as a chromatographic procedure for amino acid separation. Selected ion monitoring (SIM) of the GC effluent for ions specific to amino acids offers sensitivity comparable to that achieved with the electron-capture detector [4] and the combination of SIM analysis with stable isotopically labelled amino acids as internal standards gives a method with sensitivity and precision unmatched by other chromatographic methods [5].

Perhaps the major disadvantage in the analysis of amino acids by GC involves the effort required for isolation of these compounds from biological fluids in order to make them amenable to gas phase analysis. A large number of derivatives have been used to make amino acids sufficiently volatile and thermally stable for GC [1]. All of the derivatization procedures require prior removal of water; and considering the complexity of biological fluids with significant contributions of proteins and inorganic salts, some degree of purification is required. Ion-exchange procedures which yield the compounds of interest dissolved in a relatively large volume of aqueous solution, have been most widely used for isolation of amino acids. In studies involving many samples, the time spent in removal of water, following ion-exchange purification of the biological samples can become very inconvenient. In addition, amino acids labelled with isotopes in structural positions labile to exchange, present the possibility of loss of the isotopic label under conditions of prolonged exposure to aqueous solutions of low pH encountered in many ion-exchange procedures.

We report in this communication the development of a procedure for isolation of amino acids from biological sources which avoids the problems associated with ion-exchange purification of amino acids. The procedure is rapid, suited to the processing of large numbers of samples and delivers the amino acids in a form eminently suited for GC and GC—MS analysis. The procedure was developed primarily to facilitate isolation of amino acids under conditions which would avoid problems associated with the chemical instability of the catechol nucleus and with the possibility of loss of deuterium atoms in the aromatic ring of phenol- and catechol-amino acids.

EXPERIMENTAL

Reagents

Reagents and solvents were obtained from commercial suppliers and used without further purification. $H_2{}^{18}O$, 99 atom% ^{18}O was obtained from Mound Laboratories (Miamisburg, Ohio, U.S.A.) and used for synthesis of ^{18}O-carboxyl-labelled amino acids [$^{18}O_2$] L-dopa, [$^{18}O_2$] 3-O-methyldopa, and [$^{18}O_2$]-tyrosine by a procedure published elsewhere [6].

Blood samples from patients receiving L-dopa were collected from an indwelling catheter with a heparin lock after sufficient blood had been withdrawn to insure that dilution by the heparin solution was avoided. The blood

was transferred to a heparinized tube and immediately placed on ice. Within 30 min, the blood was centrifuged and the plasma collected. Plasma (0.2 ml) was then pipetted into a polypropylene centrifuge tube (Eppendorf) containing internal standards and 0.05 ml 3 *N* HCl; then frozen for subsequent analysis or analyzed immediately according to the procedure diagramed in Fig. 1.

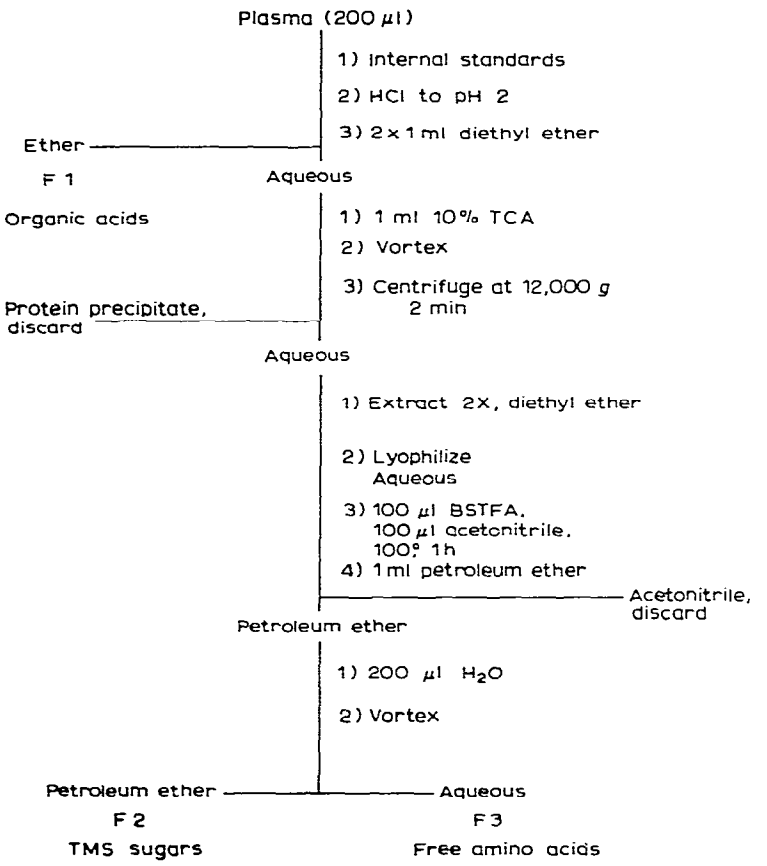

Fig. 1. Flow diagram for the sequential isolation of organic acids and non-polar neutrals (F1), trimethylsilyl ethers of polyhydroxy compounds (F2), and bases and amphoteric compounds (F3) from physiological fluids.

Procedure

The procedure for the isolation of amino acids is outlined in Fig. 1 and for the purpose of illustration, plasma is the physiological fluid. A small, precise volume of plasma (200 µl) which already contains internal standards and enough mineral acid to reduce the pH to below 2 is extracted twice with 1 ml of diethyl ether. The diethyl ether layers are removed and contain extractable organic acids (F1). The aqueous layer is then treated with 1 ml of 10% trichloroacetic acid (TCA) which precipitates plasma proteins after vigorous shaking. The capped tube is centrifuged at high speed (12,000 *g*) for 2 min in an Eppendorf Microfuge to pellet the proteins. The supernatant is separated and

extracted twice more with diethyl ether to remove TCA. It is essential to remove all the TCA. The clear, aqueous solution which is in a glass culture tube is lyophilized to complete dryness, and treated with 100 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 100 μl of acetonitrile. The test-tube is sealed with a PTFE-lined cap and heated to 100° for 1 h in a heating block. The derivatization medium is briefly cooled and treated with 1 ml petroleum ether (b.p. 35—60°). Two layers result and the upper layer is transferred to a clean culture tube. Distilled water (200 μl) is added to this layer and the two-phase system vortexed for 30 sec. The petroleum ether (F2) is immediately removed from the small volume of water. This water layer contains underivatized amines and amino acids (F3) and can be further lyophilized to complete dryness and rederivatized with 25 μl BSTFA and 25 μl acetonitrile at 100° for 1 h. This solution or aliquots thereof can be directly injected into the gas chromatograph.

The procedure in Fig. 1 yields three fractions — organic acids and nonpolar neutrals (F1), polar neutrals (F2), and amines and amino acids (F3). For some applications, complete removal of sugar contaminants in the amine-containing fraction may be desirable and can be effected by a slight modification of this procedure (Fig. 1). After lyophilization of the water backwash (F3), rederivatization with BSTFA is carried out for 5 min at 100°, but without acetonitrile. Petroleum ether extraction will then remove any sugars not completely removed previously. Amines and amino acids are not efficiently derivatized in BSTFA alone and are not soluble in petroleum ether.

Apparatus

GC—MS analysis of the fractions produced in the procedure in Fig. 1 were carried out with a Finnigan 9500 gas chromatograph (Sunnyvale, Calif., U.S.A.) interfaced via an all-glass jet separator and transfer line to a Finnigan 3200 quadrupole mass spectrometer. Mass spectral data generated by monitoring the GC effluent were acquired and stored for later analysis by a Finnigan Model 6000 data system which also controlled the mass spectrometer operating parameters. GC was carried out on 3% SE-30 on Supelcoport 100—120 mesh. Helium carrier gas flow-rate was 30 ml/min. Injector, separator oven and transfer line temperature was maintained at 250°. All mass spectra were obtained under electron impact conditions at 70 eV.

Quantitation of tyrosine, dopa and 3-O-methyldopa was done isothermally at 210° by SIM analysis. With [$^{18}O_2$]L-dopa, [$^{18}O_2$]3-O-methyldopa, and [$^{18}O_2$]tyrosine as internal standards, the mass spectrometer was focused on ions at *m/e* 218 for unlabelled dopa, 3-O-methyldopa, and tyrosine (this is a common ion for all three amino acids which retains the carboxyl ^{18}O atoms) [7] and at *m/e* 222 for [$^{18}O_2$]tyrosine, [$^{18}O_2$]dopa and [$^{18}O_2$]3-O-methyldopa. Under computer control, the mass spectrometer was rapidly switched to acquire those three ions and generate an ion profile of abundance versus time as the compounds eluted from the gas chromatograph. When the analysis was terminated, the computer was used to integrate the areas under each of the ion profile curves whose identity had been established by known retention time. The ratios of the ion profiles of interest (e.g. *m/e* 218/222) were then compared to a standard curve which had been generated using known amounts of

each amino acid in distilled water and treated along with the plasma samples. The MS analysis of each plasma sample was complete in approximately 5 min. While data are not presented, the same procedure was also followed for quantitation of plasma aromatic amino acids, only using deuterium-labelled [$^2H_3$]-L-dopa, [$^2H_2$] tyrosine, and [$^2H_3$] 3-O-methyldopa. Since the deuterium atoms were in the aromatic ring of the internal standard amino acids, the mass spectrometer was set to record ion abundances at *m/e* 179, 181, and 182 to measure the labelled and unlabelled amino acids [7].

RESULTS AND DISCUSSION

Trimethylsilylation with a strong TMS donor such as BSTFA results in substitution of a TMS group for the active proton from such moieties as alcohols, carboxylic acids, amines and some amides [8]. The derivatives formed are non-polar compounds soluble in non-polar organic solvents, often in sharp contrast to the parent compounds. This non-polarity and increased volatility have made them useful derivatives for GC analysis, but their instability to hydrolytic conditions has imposed restrictions of anhydrous conditions upon preparation and storage. The hydrolytic instability, however, is different for each kind of TMS function, and it is this differential hydrolytic stability which forms the basis for the amino acid isolation scheme presented in Fig. 1. The basis for the purification procedure is that TMS ethers are much more stable to water hydrolysis than are TMS amines or TMS esters of carboxylic acids. Polyhydroxy TMS ethers such as sugars will not be hydrolyzed back to the polar sugar by brief exposure to water and will remain soluble in a non-polar solvent such as petroleum ether. TMS amines and TMS carboxylic acids will, however, be quickly hydrolyzed to free amines and acids and will no longer be soluble in such a non-polar solvent.

Fully silylated serine has been examined in some detail with respect to its solvolytic stability [9] and serves as an illustration of differential stability of TMS derivatives. Hydrochloric acid in diethyl ether cleaved only the N—TMS bond, while water or alcohol caused rapid solvolysis of both amino and carboxyl TMS groups. The same study also reported that alcoholysis of fully silylated tyrosine and hydroxyproline caused loss of carboxyl and amine TMS groups only, but did not cleave the TMS ethers.

The relative stability of TMS derivatives to water hydrolysis in the purification of amino acids is illustrated in Fig. 2A and B. Fig. 2A is a computer reconstructed total ionization plot of a mixture of amines, amino acids and sugars which was lyophilized, derivatized with BSTFA and acetonitrile, and subjected to temperature-programmed GC—MS. The components were identified by their mass spectra. Major components seen in the chromatogram are the sugars and citric acid. Only by utilizing the MS information was it possible to determine that tyrosine co-eluted with a hexose component. After water extraction it was apparent that the only components of the mixture to remain in the petroleum ether were the sugars and citric acid. There were no amino acids apparent even when the mass spectral data files were searched for specific ions. The amines were also removed from the petroleum ether by water hydrolysis and extraction. The amino acids and amines were efficiently extracted into

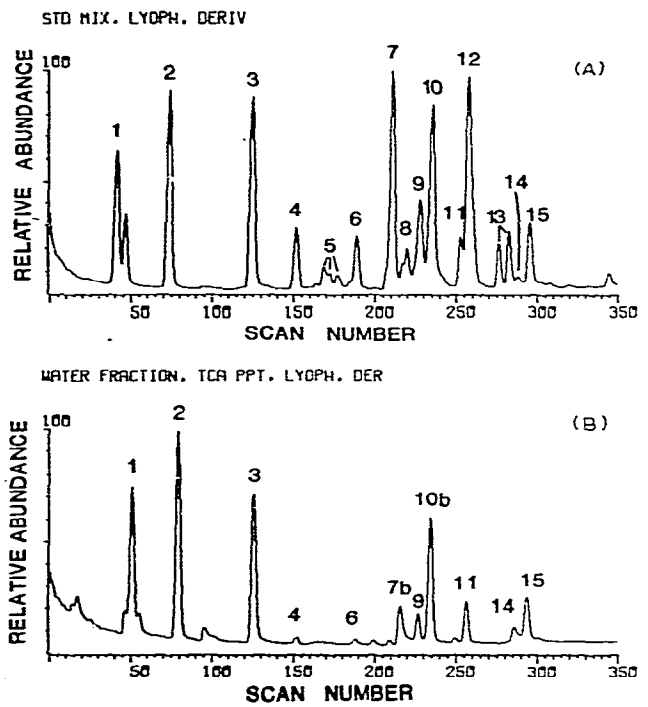

Fig. 2. Total ionization current (TIC) plots of a standard mixture of amines, amino acids and sugars. (A) TIC plot of the mixture lyophilized, derivatized and subjected to GC—MS analysis directly. (B) TIC plot of the water back-wash fraction (F3) lyophilized and derivatized. Individual components were identified by their mass spectra as: 1 = leucine; 2 = serine; 3 = α-aminobutyric acid + aspartic acid; 4 = tyramine-diTMS; 5 = ribose; 6 = 3-methoxytyramine-diTMS; 7 = citric acid; 8 = hexose; 9 = tyramine-triTMS; 10 = tyrosine + hexose; 10b = tyrosine; 11 = 3-methoxytyramine-triTMS; 12 = hexose; 13 = hexoses; 14 = tryptamine-diTMS; 15 = tryptophan + tryptamine-triTMS.

the water, as shown by Fig. 2B, which is the water back-wash (F3) after lyophilization and re-derivatization. There are no sugars apparent in the water fraction and the amino acids and amines are now free of these contaminants. Efficient mixing of the water with the petroleum ether fraction is essential for good extraction of amino acids and amines into the water. Recovery experiments with radiolabelled tyrosine and galactose demonstrated that the 30-sec period of vortex mixing recommended in Fig. 1 was sufficient to extract over 90% of the tyrosine from a derivatized plasma sample, while leaving the derivatized galactose exclusively in the petroleum ether phase. Shorter periods of vortex mixing resulted in proportionately less recovery of tyrosine. With more efficient mixing procedures, such as vigorous shaking of capped tubes, the mixing time can be reduced, but vortexing was found to be more convenient for handling large numbers of samples.

After protein precipitation, acid extraction, lyophilization and trimethylsilylation, major constituents of any plasma sample are hexoses. Fig. 3A is the reconstructed chromatogram of the petroleum ether-soluble TMS derivatives of components in a human plasma. Since amino acids are present, typical-

ly at 1—10 μg/ml in plasma, the specificity of the mass spectrometer is such that one could conceivably perform quantitative analysis of some amino acids by carefully selecting ions characteristic of each amino acid which are not present in the sugars. GC analysis alone, however, would not be possible for any amino acids which eluted in the region of the sugars. For our studies, it was desirable to use ^{18}O-labelled analogs of tyrosine, dopa and 3-O-methyl-dopa to monitor an ion common to all three amino acids which contained the oxygen-18 atoms, that being *m/e* 218 and *m/e* 222 for the ^{18}O-internal standards. As demonstrated in Fig. 3B and C, however, *m/e* 218 was a major ion in the contaminating sugars, arising as a natural isotope peak from an ion at *m/e* 217, which is an abundant ion characteristic of TMS-carbohydrates [10]. After removal of the sugars by the procedure of Fig. 1, the amino acid fraction was clean enough for both qualitative and quantitative analysis, mon-

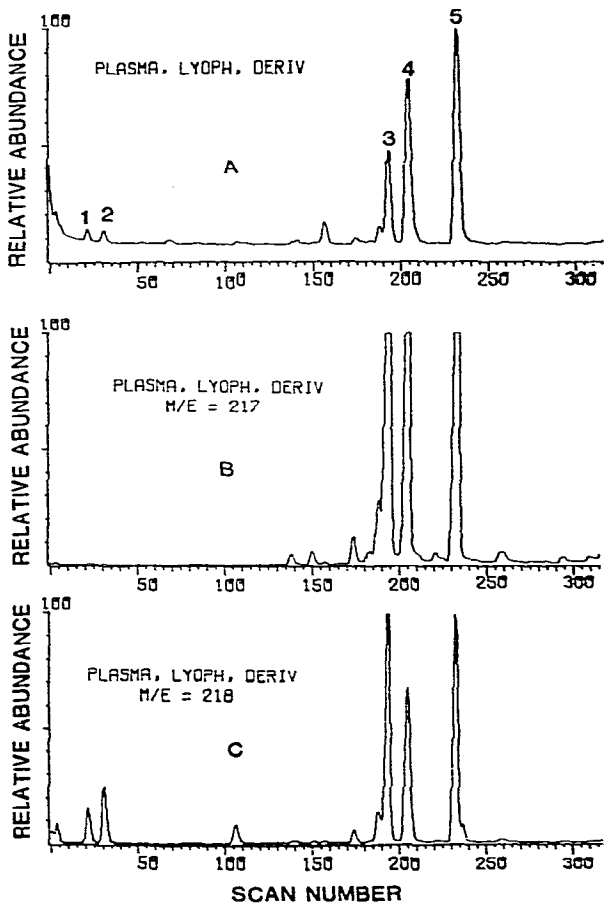

Fig. 3. Computer-reconstructed TIC plot (A) and mass chromatograms (B and C) for ions characteristic of TMS carbohydrates (*m/e* = 217, B) and TMS amino acids (*m/e* = 218, C). 200 μl of a human plasma sample were acidified with hydrochloric acid and deproteinized with TCA; the TCA was removed by ether extraction; the aqueous fraction was lyophilized to dryness, derivatized with acetonitrile and BSTFA and analyzed by GC—MS. Peaks were identified by their mass spectra as trimethylsilyl derivatives of: 1 = serine; 2 = threonine and 3—5 = hexoses.

itoring ions of *m/e* 218 and 222. Fig. 4 is the chromatogram of the water back-wash (F3) of the same sample of plasma shown in Fig. 3A. The major constituents of this fraction are now amino acids. If necessary, the remaining sugars can be removed by the slight modification described in Procedure. Since many amino acids are present in plasma in relatively high concentrations, it is obvious that any of these amino acid components of plasma can be measured using a small (200 μl) volume of plasma when the sugar contaminants are removed.

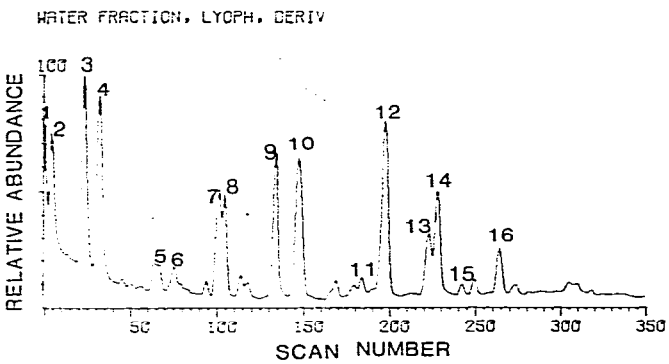

Fig. 4. Computer-reconstructed TIC plot of the water back-wash fraction (F3) of the plasma sample illustrated in Fig. 3, derivatized with BSTFA and acetonitrile and analyzed by GC—MS. Components were identified by their mass spectra as trimethylsilyl derivatives of 1 = Ile, 2 = Leu, 3 = Ser, 4 = Thr, 5 = Met, 6 = Asp, 7 = Phe, 8 = Glu, 9 = Gln, 10 = Lys-3-TMS, 11 = Hexose, 12 = Tyr and Lys-4TMS, 13 = Hexose, 14 = 3-O-methyldopa, 15 = dopa and 16 = Trp.

Fig. 5 illustrates application of this procedure to the analysis of 31 consecutive plasma samples from a Parkinsonian patient treated with L-dopa over the course of one day. Plasma samples were drawn at half-hourly intervals and then treated as described in Procedure. The total time required to prepare these samples by the described procedure for GC—MS analysis was less than one man-day.

Main contributions of this method to amino acid isolation are related to the speed, specificity, and to the mild conditions of the procedure. The method is essentially a class separation and produces three distinct fractions which are available for further analysis: acids, neutrals, and bases and amphoterics. The thrust of our work has been concerned with amino acid analysis, but the method also provides a possible route to convenient analysis of carbohydrates in biological fluids. The avoidance of ion-exchange procedures or strongly basic or acidic conditions should ensure that loss of labile isotopes would be minimized. The amino acids isolated as fraction F3 can be re-derivatized with any reagent desired, so that the method is applicable to studies with electron-capture detectors or for other specialized techniques. The method appears to be suited to a wide variety of biological fluids and we have successfully used the procedure to examine amines and amino acids in urine, human vitreous body and cerebrospinal fluid, in addition to plasma.

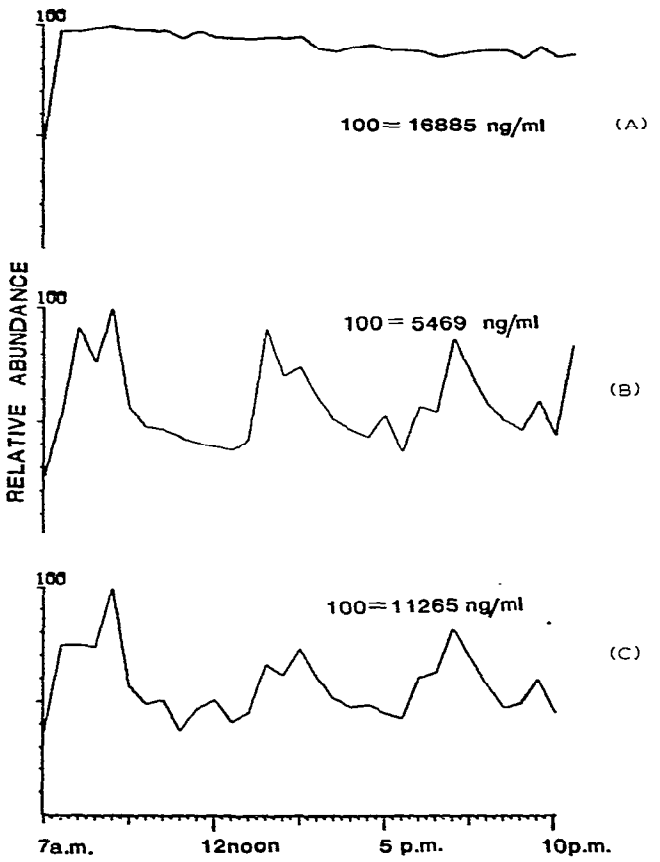

Fig. 5. Normalized plots of plasma concentration of (A) 3-O-methyldopa, (B) dopa and (C) tryptophan in a patient receiving chronic L-dopa therapy. L-Dopa was given at 7.00 a.m., 12 noon, 5.00 p.m. and 10.00 p.m. Blood samples were obtained at half-hourly intervals and the plasma amino acids were isolated as described in Fig. 1. Quantitations were performed by SIM analysis of the GC effluent, with the mass spectrometer focused to record ions of m/e = 218 and m/e 222 for the endogeneous and $^{18}O_2$-carboxyl-labelled internal standards, respectively. Ratios of the area of the ion profile curves for m/e = 218 and m/e = 222 were calculated by the computer and compared to a standard curve which was generated during the course of the analysis for each of the amino acids. Normalization was performed relative to the plasma sample of highest concentration for each amino acid, and the normalization value is shown for each compound.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Institutes of Health, AA-03527 and NS-09199, a Career Development Award HD 00128 (R.C.M.) and a training fellowship GE-07063 (K.L.C.) in Clinical Pharmacology.

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