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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ON-LINE FLOW-THROUGH RADIOACTIVITY DETECTOR SYSTEM FOR ANALYZING AMINO ACIDS AND METABOLITES LABELED WITH NITROGEN-13

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SUMMARY

A flow-through radioactivity detector was used for the high-performance liquid chromatographic determination of amino acids and other nitrogenous substances labeled with ^{13}N, a short-lived ($t_{1/2}$ 9.96 min) positron-emitting radionuclide. ^{13}N-Labeled compounds were analyzed using cation, anion and amino columns, or as the *o*-phthaldialdehyde derivative on an ODS column. Use of column-switching valves and a high-performance liquid chromatographic system with a quaternary eluting capability permits two to three 20-min analyses of labeled samples from a single ^{13}N experiment to be carried out on different columns using a binary or a single mobile phase. Radioactivity in liver metabolites was quantified using an on-line flow-through monitor with data processing capability for integrating peaks and correcting for radioactivity decay. As an example, 1 min following an L-[^{13}N]glutamate injection via the hepatic portal vein, 77% of the label in the liver was in a metabolized form; at least ten labeled products were formed.

INTRODUCTION

Amino acids labeled with the short-lived, positron-emitting radionuclides ^{13}N ($t_{1/2}$ 9.96 min) and ^{11}C ($t_{1/2}$ 20.4 min) are utilized in biomedical research to study

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physiological processes in the living organism. The changes of distribution of radioactivity with time are measured using external scanning procedures such as gamma cameras or positron emission tomography (PET). A recent review discusses the advantages and uses of ^{13}N as a biochemical tracer [1]. External scanning devices measure the quantity of radioactivity within organs or tissues but do not reveal the chemical identity of the label. The interpretation of label distribution in vivo requires chemical determination of the metabolites formed by the tissues from the administered radiolabeled amino acid in an appropriate animal model. High-performance liquid chromatography (HPLC) is ideally suited for the identification of these metabolites labeled with short-lived isotopes. The rapid separation and analyses of metabolites labeled with ^{13}N in theory may be carried out by HPLC using ion exchange, amino columns or by reversed phase with derivatization of the labeled amino acids. In contrast to precolumn derivatization with dansyl chloride [2] or phenylthiohydantoin [3], *o*-phthaldialdehyde (OPA) derivatization [4] can be carried out under the time constraints imposed by short-lived radionuclides; OPA derivatization has been used for studies with ^{13}N-labeled amino acids [5]. The radioactive eluates can be monitored by either fraction collection followed by counting of radioactivity in a gamma counter [6–8], or by use of an on-line flow-through radiation detector. The present paper describes an HPLC system for analyzing tissue extracts containing ^{13}N-labeled metabolites and an on-line flow-through detector for measuring the radioactivity. Comparison of the chromatograms of labeled metabolites obtained from an extract of liver that had been infused with L-[^{13}N]glutamate and analyzed on different columns is reported.

EXPERIMENTAL

Chemicals

Reagent-grade potassium phosphate, 2-mercaptoethanol and tetrahydrofuran were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Spectrometric-grade methanol and acetonitrile were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). HPLC-grade sodium acetate was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Fluoropa was obtained from Dionex (Sunnyvale, CA, U.S.A.) and either used directly, or in some cases, following recrystallization (four times) from ethanol. Amino acids that were used as standards and monobromotrimethylammoniobimane (qBBr) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Urease powder, type IX, from jack beans, 50–80 U/mg; glutaminase A, grade V, from *Escherichia coli*, 25 U/mg; L-alanine dehydrogenase in glycerol, from *Bacillus subtilis*, 30 U/mg; L-amino acid oxidase, type IV, from *Crotalus adamanteus*, 9 U/mg; catalase, from bovine liver, 30 000–40 000 U/mg; and argininosuccinate lyase, from bovine liver, type IV, 0.5 U/mg, were obtained from Sigma (St. Louis, MO, U.S.A.). Glutamate dehydrogenase in glycerol, from beef liver, 120 U/mg, was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Apparatus

The HPLC system consisted of a Series 4 pump with a microprocessor-controlled quaternary solvent system for gradient elution (Perkin-Elmer, Wilton, CT, U.S.A.). The mobile phases were degassed with high-purity helium gas and kept pressurized at ca. 50 kPa. Samples were introduced onto the column by an injection valve equipped with a 20-μl sample loop (Rheodyne Model 7125-S, Cotati, CA, U.S.A.). Column selection was achieved using six-port valves (Rheodyne Model 7060) placed before and after the columns. This system permitted two to three 20-min analyses of ^{13}N-labeled metabolites utilizing different columns and isocratic or gradient elution.

Chromatographic conditions

The following columns were connected to the six-port valves and used for chromatographic separations: Partisil 10 SAX and Partisil 10 SCX, 10 μm, 250×4.6 mm I.D. (Phenomenex, Palo Verdes Estates, CA, U.S.A.); Ultrasphere ODS, 5 μm, 150×4.6 mm I.D. (Altex, Berkeley, CA, U.S.A.); RSIL-NH_2, 10 μm, 250×4.6 mm I.D. (Alltech, Deerfield, IL, U.S.A.). A guard column (Aquapore CX-300, 10 μm, 30×4.6 mm I.D., Brownlee Labs., Santa Clara, CA, U.S.A.) was placed before the SCX column and an Ultrasphere ODS pre-column (45×4.6 mm, 5 μm, Altex) before the ODS column. All columns were equilibrated with appropriate buffers and solvents and maintained at room temperature.

The conditions for separating metabolites with each column were as follows: for the SCX column, the first eluting buffer was 5 m*M* potassium phosphate–hydrochloric acid (pH 2.55). After 8 min the buffer was automatically switched to 20 m*M* potassium phosphate–hydrochloric acid (pH 3.5) and the column was eluted for a further 12 min [6,8]. The flow-rate was 1 ml/min. For the SAX column, the eluting buffer was 5 m*M* potassium phosphate–hydrochloric acid (pH 3.5) for 20 min. The flow-rate was 1 ml/min [7]. For the RSIL-NH_2 column, the mobile phases consisted of 10 m*M* potassium phosphate (pH 4.3) (solvent A) and acetonitrile–water (500:70, solvent B) [9]. The proportion of solvent A was increased from 15 to 75% in 25 min with a concave curve No. 4 pattern and thereafter maintained at 75% for 10 min, by which time the radioactivity in a typical ^{13}N experiment had reached background. The flow-rate was 1 ml/min. The column was re-equilibrated by returning solvent A to 15% in 5 min with a linear gradient and then maintaining the concentration of solvent A at this level for another 10 min. For the ODS system, the mobile phases consisted of methanol (solvent C) and tetrahydrofuran–methanol–100 m*M* sodium acetate buffer (pH 7.2) (5:95:900, solvent D) [4]. A linear gradient from 10 to 80% of solvent C was run for 15 min at a flow-rate of 1.5 ml/min.

The second six-port valve was connected from each column directly to an on-line flow-through radioactivity monitor/analyzer (Ramona-D, IN/US Service, Fairfield, NJ, U.S.A.). The flow-through gamma detector had a cell body with an external scintillator (through which an empty PTFE tube passed) coupled to two photomultiplier tubes and associated electronics. The detectors used in the present experiment were either equipped with a cerium-activated lithium glass

(cell volume 300 μl) or with a plastic scintillator (cell volume 200 μl). The plastic scintillator detector was used in all the HPLC experiments.

The chromatographic profiles obtained with the Ramona-D were constructed using a program (Nuclear Interface) for the Apple IIe Computer. This program is equipped with on-line decay correction and a curve smoothing option using the conventional sliding mean or cubic fold data smoothing [10]. All chromatograms shown were smoothed using the sliding mean with an eleven-point grade for smoothing. The peaks in the chromatograms are quite broad due to the volume of the flow cell of the detector (200 μl).

After passage through the radioactivity monitor, samples were collected with a Cygnet fraction collector (ISCO, Lincoln, NE, U.S.A.). In some experiments a fluorometer (Fluorometer III, LDC, Miami, FL, U.S.A.) was placed after the radioactivity monitor to measure fluorescence of OPA derivatives.

The counting efficiencies of different scintillators for ^{3}H and ^{14}C are reported to be between 45 and 95% for the Ramona-D [11]. Because ^{13}N produces high-energy (511 keV) annihilation photons it was important to ascertain the counting efficiency in this system. Aliquots (200 μl) of ^{13}N-labeled samples were placed in a plastic scintillator flow cell and in a cesium iodide well counter and counted. The true activity of the ^{13}N-labeled sample in the well counter was calculated based on the counting efficiency of a calibrated ^{137}Cs source (29.9% counting efficiency) in the well detector.

To determine the optimal window settings for both detectors, the energy spectrum of ^{13}N was measured by counting a ^{13}N-labeled sample at different window settings. A window width of 2.5–5.0% of full scale was used. While the energy spectrum resembled the spectrum from a sodium iodide or cesium iodide detector, no summation peaks (1.02 MeV) were observed in the spectrum from either the cerium-activated lithium glass or plastic scintillators. This finding suggests that the flow-through detectors are not thick enough to detect such high energies. The photopeak of ^{13}N had a FWHM (full width at half maximum, a measure of energy resolution) of 13 and 24% for the cerium-activated lithium glass and plastic cells, respectively. In comparison, we found that ^{99m}Tc (a commonly used radionuclide in nuclear medicine) which produces a 140-keV gamma radiation, has a 49% FWHM for the plastic scintillator flow cell.

Since the half-life of ^{13}N is 9.96 min, the decay occurring during a 20-min HPLC separation is considerable. Thus, we checked the decay correction feature by filling the flow cell with a ^{13}N-labeled sample and counting it periodically over a 32-min interval.

Many laboratories do not have a flow-through gamma detector as part of an HPLC setup, but may have automatic gamma counters capable of counting samples in test tubes. Chromatograms generated from the Ramona-D detector were compared to those obtained by collecting samples every 18 s. The collected samples were each counted for 20 s in a gamma counter. The Ramona-D detector was set for an integration time of 2 s compared to a 20-s counting interval on the gamma counter. After passage through the Ramona-D, the radioactivity in the collected fractions was determined with a Packard Auto-Gamma scintillation

trary starting time and a ^{13}N-metabolite profile (counts in each fraction versus retention time) was plotted. The percentage of the total radioactivity injected onto the column in each peak was determined from the integral.

o-Phthaldialdehyde derivatization procedure

OPA (50 mg) was dissolved in methanol (1.25 ml), 50 μl of 2-mercaptoethanol and 11.2 ml of 0.4 *M* sodium borate (pH 9.5) were then added and the solution was thoroughly mixed. The solution was stored in the dark and allowed to stand for 24 h before use. 2-Mercaptoethanol (10 μl) was added to the solution every other day for two weeks after which the solution was discarded [12].

A 5-μl aliquot of a ^{13}N-labeled sample containing less than $1 \cdot 10^6$ counts was mixed with 5 μl of 2% sodium dodecyl sulfate solution in 0.4 *M* sodium borate (pH 9.5). To this were added 5 μl of the OPA derivatization solution. After allowing the reaction to proceed for 1 min (except where noted), 10 μl of 0.1 *M* potassium phosphate buffer were added and a sample (20 μl) of the mixture was analyzed by HPLC.

^{13}N-Labeled compounds

^{13}N-Labeled ammonia was produced in a CS-15 cyclotron (Cyclotron, Berkeley, CA, U.S.A.) by the p,α reaction on water and subsequent reduction of ^{13}N-labeled nitrate and nitrite with Devarda's alloy and sodium hydroxide [13]. Typical yields of [^{13}N]ammonia were 300–400 mCi in a final volume of 3 ml, with a specific activity of ca. 1 Ci/μmol at the end of synthesis. L-[^{13}N]Glutamate was prepared from labeled ammonia using immobilized glutamate dehydrogenase [14]; typical yields were 75–130 mCi in 6 ml with a specific activity of ca. 0.5 Ci/μmol at the end of synthesis. L-[^{13}N]Glutamate (10–30 mCi) was perfused into the hepatic portal vein of a ketamine–diethyl ether anesthetized Sprague–Dawley rat through a 30-gauge catheter for 20 s. At 1 min from the start of injection, a sample (ca. 100 mg) of liver was excised and homogenized in a three-fold excess of ice-cold 1% picric acid and centrifuged in a microfuge (Beckman) for 30 s at 12 000 *g*. The supernatant, which contained the labeled metabolites (the most important of which are urea, aspartate, glutamate, glutamine, alanine and ammonia), was passed through a 0.22-μm Millex-GS filter (Millipore, Bedford, MA, U.S.A.) and was analyzed by HPLC using a cation, anion or amino column.

Identification of ^{13}N-labeled metabolites

Peak retention times were compared with those of unlabeled standards by injecting 50 μmol of standard and collecting 30-s fractions. The retention time of urea was determined by spotting aliquots onto a Whatman No. 1 filter paper and spraying with Ehrlich's reagent [1% dimethylaminobenzaldehyde in ethanol–2 *M* hydrochloric acid (1:1)]; a yellow spot denotes the presence of urea. Ammonia was determined by Nesslerization. The retention times of the amino acids were determined by spotting 50 μl of the fractions onto a filter paper and spraying with ninhydrin (the presence of an amino acid is signified by a blue spot) or by measuring absorbance at 200 nm with a UV monitor. By considering the most likely metabolites of L-[^{13}N]glutamate in liver (urea cycle intermediates, products of

transamination reactions, γ-glutamyl cycle intermediates and glutamine) and by comparing the retention times obtained from standards, the possible component(s) of each peak eluting from the SCX column were deduced to be as follows: peak 1 (eluting at ca. 5 min), urea; peak 2 (eluting at ca. 6 min), aspartate and γ-glutamylcysteine; peak 3 (eluting at ca. 6.5 min), glutamate and glutathione; peak 4 (eluting at ca. 7.5 min), glutamine and other neutral amino acids; peak 5 (eluting at ca. 8.5 min), alanine and citrulline; peak 6 (eluting at ca. 17.5 min), ammonia and argininosuccinate. The further identification and quantitation of metabolites were carried out as follows: the HPLC effluent was collected in 6-ml tubes every 18 s. Each tube was counted to determine the peaks of radioactivity and to match these peaks against the HPLC readout. Since the dead-time between the column and the fraction collector was known (36 s), the peak of interest was easily obtained from the collected fractions.

Peak 1. [^{13}N]Urea was identified by release of [^{13}N]ammonia after treatment with urease. Phosphate buffer (pH 8.0, 1 *M*, 50 μl) and 250 U of urease were added; the mixture was incubated for 10 min at 37°C. The sample was applied to a Dowex-50 (Tris, 2.5×0.5 cm) column and the column was washed with 6 ml of water. [^{13}N]Ammonia remained on the column and represented label originally present as [^{13}N]urea [6].

Peak 2. L-[^{13}N]Aspartate and ^{13}N-labeled γ-glutamylcysteine were measured by separating the qBBr adduct of γ-glutamylcysteine from aspartate. Tris–HCl (0.1 *M*, pH 8.2, 20 μl) and 1 mg of qBBr, which was introduced as a convenient adduct for the HPLC determination of thiols of biological interest [15], were added and the solution was incubated for 5 min at 37°C. The solution was applied to a Dowex-50 (Tris, 2.5×0.5 cm) column and washed with 6 ml of water. The radioactivity in the pass-through and water wash represented L-[^{13}N]aspartate. The activity remaining on the column represented the qBBr adduct of ^{13}N-labeled γ-glutamylcysteine (qBBr has a positively-charged quaternary nitrogen that interacts strongly with the Dowex-50 resin).

Peak 3. L-[^{13}N]Glutamate was determined by deamination of a sample of peak 3 with glutamate dehydrogenase followed by trapping the released [^{13}N]ammonia. Tris–acetate (1 *M*, pH 8.4, 50 μl), 10 μl of 100 m*M* NAD^+, 5 μl of 1 *M* hydrazine hydrate and 200 μg of enzyme were added and the solution allowed to incubate for 10 min at 37°C. The sample was applied to a Dowex-50 (Tris, 2.5×0.5 cm) column and the column was washed with 6 ml of water. [^{13}N]Ammonia remained on the column and represented label originally present in L-[^{13}N]glutamate. Since glutathione coelutes in peak 3, a separate aliquot of peak 3 was processed to correct for labeled glutathione. This aliquot was treated with qBBr and processed as described above for γ-glutamylcysteine. The radioactivity remaining on the Dowex-50 (Tris, 2.5×0.5 cm) column was due to the qBBr adduct of ^{13}N-labeled glutathione, and the pass-through and water wash was due to L-[^{13}N]glutamate.

Peak 4. ^{13}N-Labeled glutamine was determined by adding 250 μg of glutaminase to peak 4. After incubating for 10 min at 37°C the mixture was applied to a Dowex-1 (acetate, 0.5×0.5 cm) column. The column was washed with 6 ml of water and combined with the pass-through. The column was next washed with 6

ml of 1 *M* potassium chloride; the radioactivity in this fraction represented ^{13}N label in the α-amino position of glutamine. The pass-through and water wash were then applied to a Dowex-50 (Tris, 2.5×0.5 cm) column and washed with 6 ml of water. The radioactivity in this fraction was due to neutral amino acids coeluting with glutamine in peak 4. The ^{13}N activity remaining on the column represented the ^{13}N label in the amide nitrogen of glutamine [16].

Peak 5. L-[^{13}N]Alanine was determined in peak 5 by adding 1.5 U of alanine dehydrogenase, 50 μl of 1 *M* ammediol and 50 μl of 1 *M* semicarbazide–HCl (previously taken to pH 7.5 with concentrated sodium hydroxide). The mixture was incubated for 10 min at 37°C and then applied to a Dowex-50 (Tris, 2.5×0.5 cm) column. The column was eluted with 6 ml of water. [^{13}N]Ammonia remaining on the column represented radioactivity originally present in L-[^{13}N]alanine; radioactivity in the water wash represented labeled neutral amino acids that coelute with alanine.

L-[ω-^{13}N]Citrulline, which coelutes with alanine, was identified by adding L-amino acid oxidase (5 U), 50 μl of 0.1 *M* potassium phosphate, pH 7.2, and 50 U of catalase to a sample of peak 5. After incubating for 10 min at 37°C, the sample was applied to a Dowex-50 (H^+, 2.5×2.5 cm) column and the column was washed with 6 ml of water. Unreacted ^{13}N-labeled amino acids and [^{13}N]ammonia (from the oxidation of [amine-^{13}N]amino acids) remained on the column and α-keto[^{13}N]carbamidovalerate was eluted with the wash and represented label originally present in L-[ω-^{13}N]citrulline.

Peak 6. This fraction was treated with 500 μg of glutamate dehydrogenase, 20 μl of 250 m*M* α-ketoglutarate, 100 μl of 10 m*M* NADH and 0.2 U of argininosuccinate lyase. After incubating for 10 min at 37°C the sample was applied to a Dowex-50 (Tris, 2.5×0.5 cm) column and washed with 6 ml of water. The activity in the pass-through and wash represented L-[^{13}N]glutamate derived from [^{13}N]ammonia. The activity remaining on the column represented L-[ω-^{13}N]arginine derived from labeled argininosuccinate.

RESULTS

Flow-through gamma detector

A counting efficiency of 5.1% for ^{13}N was calculated for the plastic scintillator flow cell by comparing the observed sample count rate to that obtained with the calibrated well counter. Although the efficiency of the flow cell is low, the background is also very low (0–2 counts per 2 s) resulting in a good signal-to-noise ratio.

The radioactivity monitor is interfaced to a computer for data handling. This system provides a number of useful features, such as decay correction, peak integration and curve smoothing. A plot of decay-corrected counts (initially ca. 35 000 counts) versus time is linear for at least 35 min. Each data point is corrected for decay before being plotted, a useful feature when working with short-lived isotopes, such as ^{13}N.

Fig. 1 compares the profiles of ^{13}N-labeled metabolites, prepared from the same sample of rat liver after infusion of L-[^{13}N]glutamate, in HPLC eluates from a

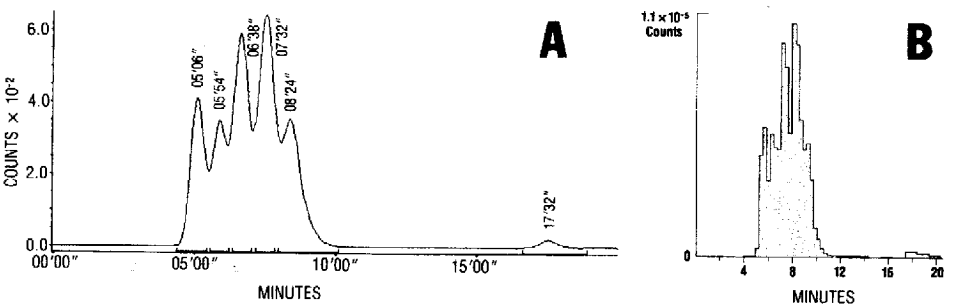

Fig. 1. Comparison of on-line detection versus fraction collection. The chromatogram shows six peaks of ^{13}N-labeled metabolites eluted from (A) an SCX column and monitored with a Ramona-D; and (B) 18-s fractions collected and the radioactivity determined in a Packard gamma counter.

cation-exchange column using on-line detection versus fraction collection. Although six peaks can be seen in each chromatogram, the resolution is clearly better in the flow-through system. These peaks were further identified enzymatically or chemically as described above. Distribution of labeled metabolites in a typical experiment was as follows (the percent radioactivity in each peak is given in parenthesis; the average composition of labeled products in each peak from a composite of several experiments is given after the hyphen): peak 1 (16.0%) — 80% urea and 20% unknown; peak 2 (14.3%) — 98% aspartate and 2% γ-glutamylcysteine; peak 3 (22.7%) — 95% glutamate and 5% glutathione; peak 4 (25.0%) — 30% amine glutamine, 14% amide glutamine and 56% other neutral amino acids; peak 5 (20.7%) — 49% alanine and 51% citrulline; peak 6 (1.3%) — 93% ammonia and 7% argininosuccinate.

In pilot experiments, HPLC separation of all of the labeled amino acids of interest by ion-exchange or amino columns was not achieved. Therefore, we tried OPA derivatization followed by reversed-phase chromatography. OPA derivatization has been used to quantify 20 amino acids within 25 min (e.g. ref. 12) and has been used for the identification of ^{13}N-labeled amino acids [5]. Ammonia is expected to be an important labeled metabolite in studies with ^{13}N-labeled amino acids. Therefore, we investigated the rate of formation of its [^{13}N]OPA derivative (unreacted [^{13}N]ammonia has a retention time of 1 min 40 s on the ODS column). After reacting with OPA for 1 and 2 min, only 11 and 33% respectively, of labeled ammonia was derivatized. At 5 min, OPA derivatization resulted in multiple peaks with the major one at 7 min 26 s, representing 44% of the total radioactivity. At 10 min, 75% of the activity was present in one peak with a retention time of 6 min 56 s. Another important metabolite in our studies is L-[^{13}N]glutamate. In Fig. 2, a 2-min OPA derivatization reaction with ^{13}N-labeled glutamate is shown. The peak, with a retention time of 6 min 32 s, corresponds to that of the fluorescent glutamate OPA derivative peak as measured on a fluorometer (retention time 6.1 min, Table I). The radioactivity under this peak accounts for 51% of the total activity applied to the column. A broad peak between 1–6 min accounts for 43% of the total measured radioactivity. A small peak occurring immediately after the glutamate peak accounts for 6% of the total radioactivity. We have not yet determined whether the non-fluorescent peaks are due to

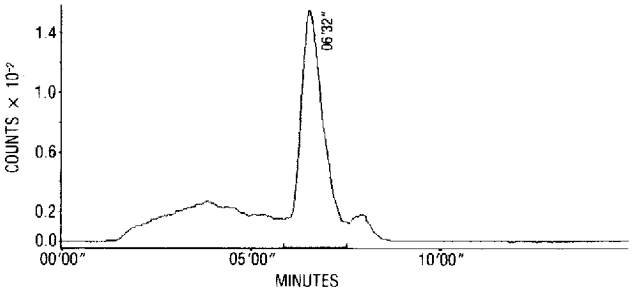

Fig. 2. Chromatogram obtained following a 2-min OPA derivatization reaction of L-[^{13}N]glutamate. Peaks of radioactivity occur before and after the major peak which coincides with the fluorescent product.

impurities, intermediates or breakdown products in the OPA derivatization reaction. However, when recrystallized (four times) OPA was used, a major peak containing 94% of the radioactivity was noted (data not shown). This finding suggests that the major portion of the counts in the ^{13}N peaks, other than the fluorescent OPA-glutamate product (49%) shown in Fig. 2, was due to the reaction of $^{13}NH_3$ with impurities in the original OPA.

Because OPA derivatization for ^{13}N analyses, even with four times recrystallized OPA, was not fully quantitative in our hands, we tried several techniques that did not require prior derivatization. Fig. 3 shows the elution patterns of labeled underivatized amino acids produced from the metabolism of L-[^{13}N]glutamate by the rat liver. The major labeled components eluting separately from the SCX column are urea, aspartate, glutamate, glutamine, alanine and ammonia. The SAX column separates labeled ammonia, a single peak containing neutral amino acids plus urea, glutamate and aspartate. The RSIL-NH_2 separates labeled urea,

TABLE I

RETENTION TIMES OF NITROGEN-CONTAINING STANDARDS ELUTING FROM DIFFERENT HPLC COLUMNS

Except for the ODS column, the retention times are those of the underivatized compounds and were determined as described in the Experimental section. In the case of the ODS column, amino acids were determined with a fluorescence monitor following a 2-min OPA derivatization.

Column	Retention time (min)					
	Urea	Asp	Glu	Gln	Ala	Ammonia
SCX	4–5	5.5–6.5	6.5–7.5	7.5–8.5	8.5–9.5	17–18
SAX	3–4	12–13	8–9	4–5	4–5	2–3
NH_2	4.8	26.4	25.9	15.6	14.2	27–30
ODS	n.d.★	3.8	6.0	8.1	11.0	n.d.★★

★n.d. = not determined.

★★Ammonia has been detected and quantified as the OPA derivative [12] but the fluorescent peak is much less intense than that of the amino acid OPA compounds. We were unable to consistently obtain a single fluorescent peak and analysis of $^{13}NH_3$-OPA reactions revealed several peaks of radioactivity (see Results).

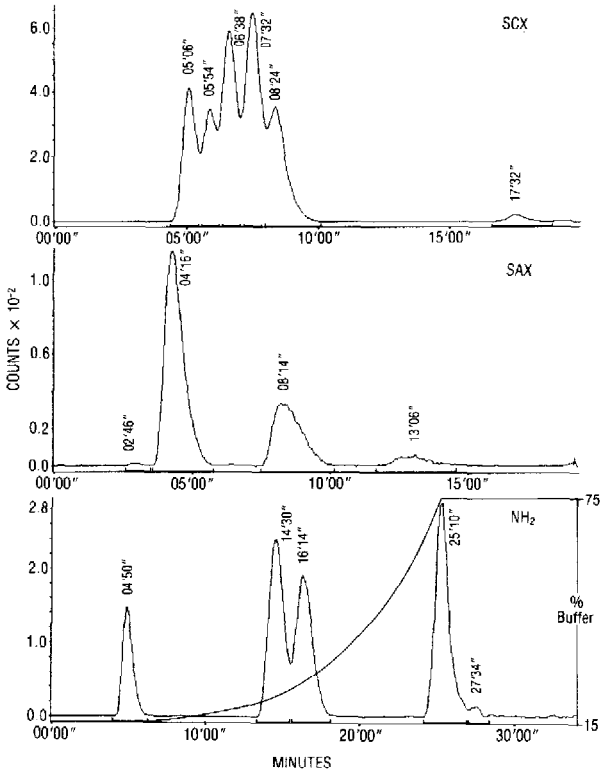

Fig. 3. Separation of ^{13}N-labeled metabolites using a cation (SCX), anion (SAX) or an amino (NH_2) column. In the example shown, the SCX and SAX ^{13}N analyses were carried out on the same liver sample, showing the capability of the present system for multiple analyses of ^{13}N-labeled metabolites. The amino column profile was obtained from a separate experiment. The identities of the ^{13}N-labeled metabolites and their retention times on each column are given in Table I.

alanine, acidic amino acids (glutamate plus aspartate) and ammonia. The retention times of the unlabeled standards for each column are shown in Table I.

DISCUSSION

The present study describes the use of an HPLC system coupled to an on-line flow-through detector for monitoring and identifying metabolites labeled with the short-lived positron-emitting radionuclide, ^{13}N. Previous studies [6–8,16] have been carried out using manual collection of fractions and their subsequent counting in a gamma counter. The on-line detector is more convenient, gives better resolution and provides automatic quantitation. (On-line detection has been reported as a convenient method for monitoring tritiated compounds in HPLC eluates [17].) In addition to the greater accuracy of the present method, on-line detection with decay correction provides a means for graphic display and quantitation of peaks of radioactivity.

In order to obtain adequate resolution via the manual method, fractions must be collected at short intervals. A fraction collector set at three tubes per min will

result in 60 fractions over a 20-min period. Each fraction must then be counted for 20-s intervals in the auto-gamma counter. This interval, plus a 20-s sample change over time, results in a total of 40 min to count all the fractions. Since the half-life of ^{13}N is 9.96 min, only one HPLC analysis per ^{13}N experiment is possible using fraction collection compared to two or three analyses possible with the on-line flow-through radioactivity detector. In the latter case, multiple analyses can be (a) of the same sample on different columns, (b) of different tissue specimens on the same type of columns, or (c) a combination of both.

It is generally not possible to use the same HPLC column for consecutive analyses of samples obtained from a single ^{13}N experiment; re-equilibration of the column takes too long. This problem is overcome by using switching valves that allow the second sample to be analyzed on a separate column immediately after completion of the first analysis (only the inflow tubing prior to the column must be purged).

The on-line procedure works well where a single ^{13}N peak can be unambiguously shown to be due to a single component. However, where there is a suspicion that a peak contains two or more radioactive components, fraction collection for further analyses may be necessary.

The counting efficiency for ^{13}N is only 5.1% (^{14}C and ^{3}H are counted with 45–95% efficiency [11]). Since the background is very low, we have not found the low efficiency to be a problem but a higher efficiency may be desirable when working with samples containing lower radioactivity. A system with a sodium iodide detector replacing the cerium-activated lithium glass or plastic scintillator would in theory have a higher efficiency and would resolve high-energy gamma rays better than the present detectors. Another potential drawback with the Ramona-D is the limitation of the electronic counting system. The present system registers only up to 100 000 counts per 2 s. If the count rate exceeds this value, the true count rate is either not recorded, or the machine shuts off. Thus, care must be taken when analyzing HPLC samples with high radioactivity so as not to overload the system.

Using the present on-line system we have found that the SCX column gives the best resolution to-date of ^{13}N-labeled metabolites (Fig. 3). However, this method does not separate all possible ^{13}N-labeled metabolites and further enzymatic or chemical analyses are necessary. For example, 1 min following administration of [^{13}N]glutamate to the liver, 77% of the label is present in a metabolized form. Six peaks are visible from the SCX HPLC profile; further analyses revealed the presence of at least ten ^{13}N-labeled metabolites. The ability to identify the ^{13}N components of each peak as early as possible is critical.

Derivatization of unlabeled amino acids with OPA and separation of the fluorescent product by reversed-phase chromatography provides good resolution and is very rapid. The OPA reaction essentially goes to completion [18]. Moreover, OPA itself has the property of being non-fluorescent under the conditions of the analysis. HPLC of OPA derivatives is now a routine procedure for amino acid analyses (e.g. refs. 4, 12 and 19). At first we were optimistic that OPA derivatization may provide a rapid method for ^{13}N-labeled metabolite analysis, but our experience with [^{13}N]glutamate and [^{13}N]ammonia was disappointing. More-

over, others have shown that some OPA-amino acids (e.g. GABA, glycine) break down very rapidly (e.g. refs. 20 and 21). Secondly, OPA may interact with non-amine, nitrogen-containing metabolites either not at all or in an unpredictable fashion. On the other hand Barrio and co-workers [5,22] suggest that ^{13}N-labeled amino acids in biological specimens may indeed be quantitated by HPLC analysis of the OPA derivative.

For short-term metabolism studies of nitrogen-containing compounds, ^{13}N has several advantages over ^{15}N as a tracer [1]. In the present work we have shown that the on-line system offers advantages over previously used techniques of ^{13}N-labeled metabolite analyses. With improvements in (a) counting efficiency of the detector and (b) resolution, column design and speed of HPLC analysis, we can look forward to a greater use of the biomedically important positron-emitting isotopes (^{13}N, ^{11}C, ^{18}F and even ^{15}O; $t_{1/2}$ ca. 10, 20, 110 and 2 min, respectively) as tracers in animal experiments. PET studies of human subjects require a detailed knowledge of the biochemical pathways involved in the metabolism of the administered labeled compound. Such pathways are usually inferred from animal studies. The new HPLC techniques may provide a useful adjunct to PET studies by providing detailed information on the disposition of labeled metabolites directly from the subject's body fluids.

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REFERENCES

1 A.J.L. Cooper, A.S. Gelbard and B.R. Freed, in A. Meister (Editor), Advances in Enzymology and Related Areas of Molecular Biology, Vol. 57, Wiley, New York, 1985, p. 251.
2 E. Bayer, E. Grom, B. Kaltenegger and R. Uhmann, Anal. Chem., 48 (1976) 1106.
3 D. Hawke, P.-M. Yuan and J.E. Shively, Anal. Biochem., 120 (1982) 302.
4 B.N. Jones and J.P. Gilligan, J. Chromatogr., 266 (1983) 471.
5 F.J. Baumgartner, J.R. Barrio, E. Henze, H.R. Schelbert, N.S. MacDonald, M.E. Phelps and D.E. Kuhl, J. Med. Chem., 24 (1981) 764.
6 K.C. Rosenspire, A.S. Gelbard, A.J.L. Cooper, F.A. Schmid and J. Roberts, Biochim. Biophys. Acta, 843 (1985) 37.
7 A.J.L. Cooper, S.N. Mora, N.F. Cruz and A.S. Gelbard, J. Neurochem., 44 (1985) 1716.
8 A.S. Gelbard, D.S. Kaseman, K.C. Rosenspire and A. Meister, Int. J. Nucl. Med. Biol., 12 (1985) 235.
9 R. Schuster, Anal. Chem., 52 (1980) 617.
10 A. Savitzky and M.J.E. Golay, Anal. Chem., 36 (1964) 1627.
11 G. Dietzel, GIT Fachz. Lab., 28 (1984) 909.
12 B.N. Jones, S. Pääbo and S. Stein, J. Liq. Chromatogr., 4 (1981) 565.
13 A.S. Gelbard, L.P. Clarke, J.M. McDonald, W.G. Monahan, R.S. Tilbury, T.Y.T. Kuo and J.S. Laughlin, Radiology, 116 (1975) 127.

14 A.S. Gelbard, R.S. Benua, R.E. Reiman, J.M. McDonald, J.J. Vomero and J.S. Laughlin, J. Nucl. Med., 21 (1980) 988.
15 R.C. Fahey, G.L. Newton, R. Dorian and E.M. Kosower, Anal. Biochem., 107 (1980) 1.
16 A.J.L. Cooper, J.M. McDonald, A.S. Gelbard, R.F. Gledhill and T.E. Duffy, J. Biol. Chem., 254 (1979) 4982.
17 R.F. Roberts and M.J. Fields, J. Chromatogr., 342 (1985) 25.
18 S.S. Simons, Jr. and D.F. Johnson, J. Org. Chem., 43 (1978) 2886.
19 D.C. Turnell and J.D.H. Cooper, Clin. Chem., 28 (1982) 527.
20 R.F. Chen, C. Scott and E. Trepman, Biochim. Biophys. Acta, 576 (1979) 440.
21 L.A. Allison, G.S. Mayer and R.E. Shoup, Anal. Chem., 56 (1984) 1089.
22 J.R. Barrio, R.E. Keen, D.C. Chugani, G. Bida, N. Satyamurthy and M.E. Phelps, in D.M. Wieland, M.C. Tobes and T.J. Mangner (Editors), Analytical and Chromatographic Techniques in Radiopharmaceutical Chemistry, Springer-Verlag, New York, 1986, p. 215.