Enzymatic-Chemical Preparation of Quinoxaline Derivatives from L-Amino Acids for Gas Chromatographic-Mass Spectrometric Analyses

Peter Schadewaldt,* Hans-Werner Hammen,† Udo Wendel,† and Uwe Matthiesen‡
*Diabetes Forschungsinstitut, †Kinderklinik, and ‡Spurenelementlabor,
Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

Received October 17, 1994

We report on an enzymatic-chemical method for the specific preparation of L-amino acid-derived quinoxalinols suitable for the sensitive estimation of $^{13}$C- or $^2$H-label enrichment by gas chromatography-mass spectrometry: Amino acid fractions are isolated from physiological fluids by ion-exchange chromatography. The compound of interest is converted to the corresponding 2-oxo acid by treatment with an L-amino acid dehydrogenase of the desired specificity. Reaction of the 2-oxo acid with o-phenylenediamine yields the quinoxaline derivative. Isotopic label enrichment is then determined by gas chromatographic-mass spectrometric analysis of the O-trimethylsilyl derivative using ammonia-chemical ionization mode and selected ion monitoring of the quasi-molecular ions $[M+H]^+$ and $[M+H+n]^+$ ($n =$ number of labeled positions). Details for application of various generally available L-amino acid dehydrogenases (L-ala DH, L-glu DH, L-leu DH, L-phe DH) are presented. The method was used, e.g., for serum analysis in in vivo studies on the decarboxylation rates of branched-chain L-[1-$^{13}$C]-amino acids. © 1995 by Academic Press, Inc.

In recent years, increasing availability of stable isotope labeled substrates and instrumentation for measurement of isotope enrichment in metabolites has greatly stimulated in vivo approaches for the study of metabolic fluxes in man (1). In numerous studies, stable isotope labeled L-amino acids have been used as substrates. To account for the dilution of isotope label in the L-amino acid and possibly in the derived 2-oxo acid pools of the body, gas chromatographic-mass spectrometric analyses (GC-MS) of the label enrichment in the protein-bound and/or free compound(s) of interest must be performed (1).

Among the numerous derivatization procedures suitable for gas chromatographic separation of L-amino acids (see (2,3) for reviews) and subsequent analysis by MS, two methods have been generally applied: silylation (4–7) in combination with electron-impact ionization (EI) and esterification followed by N-acylation (8–14) in combination with either chemical ionization (CI) or EI. In contrast to the variety of methods applied for GC-MS analysis of stable isotope labeled amino acids, there is a method of choice for the derived 2-oxo acids: 2-Oxo acids readily form quinoxaline derivatives by reaction with o-phenylenediamine (15). The O-TMS quinoxalinol derivatives are stable and exhibit favorable gas chromatographic properties, thus facilitating the separation of complex mixtures. Using CI, only minor fragmentation occurs which is a prerequisite for sensitive MS determination of label enrichment (16–19).

When we started studies on the effect of oral loads of L-$^{13}$C-labeled branched-chain L-amino acids on serum kinetics and whole-body oxidation rates of branched-chain compounds in healthy persons and patients with maple syrup urine disease, we found it rather unfavorable to run different GC-MS programs for the determination of $^{13}$C-label enrichment in serum L-amino and the derived 2-oxo acids. We therefore examined methods for the conversion of L-amino acids into quinoxaline derivatives.

Chemical procedures have been described (20). They are, however, unspecific and rather complicated. Thus,
we studied the use of L-amino acid dehydrogenases for the preparation of quinoxaline derivatives from L-amino acids via their corresponding 2-oxo acids.

In the present communication we report on the application of generally available L-amino acid dehydrogenases for the specific enzymatic-chemical synthesis of quinoxaline derivatives of L-alanine, branch-chain L-amino acids, L-glutamate, and L-phenylalanine from physiological fluids. Usefulness of the method for GC-MS measurement of $^{13}$C-label enrichment is demonstrated, e.g., by serum analysis after oral loading with branch-chain L-$^{[13C]}$amino acids.

**MATERIALS AND METHODS**

**Chemicals**

Unless otherwise noted, all chemicals and reagents were purchased in the highest available purity from Merck (Darmstadt, Germany) or Sigma Chemie (Munich, Germany). Coenzymes, L-alanine dehydrogenase (EC 1.4.1.1; from *Bacillus subtilis*), and L-glutamic acid dehydrogenase (EC 1.4.1.3; from beef liver) were from Boehringer (Mannheim, Germany), L-leucine dehydrogenase (EC 1.4.1.1; from *Bacillus subtilis*) and *Rho-disococcus sp.* from Calbiochem (Bad Soden, Germany).

For convenience, L-amino acid dehydrogenase activities were determined spectrophotometrically at 25°C in glycine/NaOH buffer (pH 10.0) containing EDTA (1 mmolliter), NAD$^+$ (4 mmolliter), and 10 mmolliter of the appropriate L-amino acid substrate. For assay of L-glutamic acid dehydrogenase from beef liver, the mixture additionally contained ADP (2 mmolliter) and L-glutamate concentration was 20 mmolliter. One unit is defined as the amount of enzyme activity reducing 1 μmol NAD$^+$ per minute under the above assay conditions.

L-[1-$^{13C}$]Leucine (1-$^{13C}$, 99%) was obtained from Campro Scientific (Emmerich, Germany), L-[1-$^{13C}$]valine (1-$^{13C}$, 99%) and L-[1-$^{13C}$]phenylalanine (ring-$^{13C}$, 99%) were from Promochem (Wesel, Germany) and L-amino acid dehydrogenase (EC 1.4.1.9; from *Rho-disococcus sp.*) from Calbiochem (Bad Soden, Germany).

Preparation of 13C-Enriched Controls

Control serum (Precinorm from Boehringer) was used to prepare 0.3 mmolliter with pure natural L-phenylalanine or L-leucine and pure L-[ring-$^{13C}$]phenylalanine (ring-$^{13C}$, 99%) or L-[1-$^{13C}$]leucine (1-$^{13C}$, 99%). Appropriate volumes of the stock solutions were mixed to give a calculated 1-$^{13C}$-label enrichment between 2 and 30% as given under Results. Samples of the stock solutions and the mixtures were then analyzed for label enrichment as detailed below.

In Vivo Loading Studies

After an overnight fast, a healthy volunteer and a patient with maple syrup urine disease (male, 14 years) orally ingested L-[1-$^{13C}$]valine or L-[1-$^{13C}$]leucine (38 μmol/kg body wt). Blood samples were taken from a cannula inserted into the basilic vein at 30 min and just before the load (control values) and further every 15 min over a period of 3 h.

Serum samples were analyzed for concentration and 13C-label enrichment in branch-chain compounds as described below.

Analysis of Amino and 2-Oxo Acids

For amino acid analysis, serum samples were mixed with an equal volume of Li-citrate buffer (Li$^+$, 0.1 mol/liter, pH 2.2) and protein was removed by ultrafiltration (centrifuge from Amicon, Witten, Germany). Amino acids were then determined on an automatic amino acid analyzer (LC 5001; Eppendorf-Biotronik, Maintal, Germany) (21).

The procedure for branch-chain 2-oxo acid and phenylpyruvate analysis has been described previously in detail (23). In short, 2-oxo acids in deproteinized serum samples were reacted with o-phenylenediamine to yield the quinoxaline derivatives. After solid-phase extraction on a 1-ml Cl8 column (from Baker, Groß-Gerau, Germany), the quinoxaline derivatives were quantified using reversed-phase high-performance liquid chromatography and fluorescent detection. 2-Oxohexanoate was used as an internal standard, and blanks and standard solutions were run in parallel.

For analysis of the more hydrophilic quinoxaline derivatives of pyruvate and 2-oxoglutarate, the solid-phase extraction procedure and the conditions of reversed-phase HPLC analysis had to be modified as follows: After reaction with o-phenylenediamine, the reaction mixture (1 ml) was applied onto a 1-ml C18 SPE column. After successive washes with 1 ml water and 1 ml methanol:water (1:4, v/v), the quinoxaline derivatives were eluted with 0.5 ml CH3CN:water (30%, v/v). HPLC analysis was carried out using 20% CH3CN (v/v) under isocratic conditions.
Preparation of Quinoxaline Derivatives from L-Amino Acids

For preparation of quinoxaline derivatives of L-amino acids other than glutamate, serum (0.2 ml) was mixed with 5-sulfosalicylic acid (0.5 ml, 5%, w/v). After centrifugation (10 000g, 5 min, 4°C), amino acids were separated from preformed 2-oxo acids by SPE on a 1 ml sulfonic acid SPE column (from Baker) prewashed with 1 ml methanol and 1 ml water. Acid extract (0.5 ml) was applied to the column followed by two washes with 1 ml water. Amino acids were then eluted with 0.5 ml NH4OH (2 mol/liter). After addition of 0.02 ml NaOH (2 mol/liter), the solution was evaporated to dryness under a stream of nitrogen.

In the latter procedure, hydrolysis of glutamine occurs causing interference with the determination of label in glutamate. Therefore, glutamate was first separated from glutamine as follows: 0.2 ml serum was mixed with cold (-20°C) 75% ethanol (v/v, 0.8 ml) and precipitated protein removed by centrifugation. Supernatant (0.8 ml) was applied onto a 1 ml anion-exchange SPE column (quaternary amine, acetate form; Macherey-Nagel, Düren, Germany) and directly connected to a 1 ml cation-exchange SPE column (quarternary amine, acetate form; from Baker) and glutamine (and other amino acids) removed by two washes with 1 ml water. The adsorbed acidic amino acids were eluted with 0.5 ml acetic acid (2 mol/liter). When preformed 2-oxo acids in the acid eluate were initially decarboxylated by treatment with hydrogen peroxide, problems occurred with the HPLC analysis of quinoxaline derivatives. Therefore, interfering compounds were removed from the acid eluate by the cation-exchange chromatographic procedure described above.

For treatment with L-amino acid dehydrogenases, the dry residue was dissolved in 0.1 ml HCl (0.4 mol/liter) and 0.15 ml conversion mixture was added containing glycine/NaOH buffer (0.33 mol/liter, pH 10.0), NAD⁺ (13 mmol/liter), and enzyme activity as appropriate. Enzyme activities in mmol x min⁻¹ x liter⁻¹ amounted to L-alanine dehydrogenase, 1.7; L-glutamic acid dehydrogenase, 1.5; L-leucine dehydrogenase, 0.5; and L-phenylalanine dehydrogenase, 0.07. After 30 min of incubation at 37°C, 0.5 ml HClO₄ (0.8 mol/liter) was added and enzyme protein removed by centrifugation (10 000g, 5 min, 4°C). The L-amino acid derived 2-oxo acids in the acid supernatant (0.5 ml) were then reacted with o-phenylenediamine (10 g/liter in 2 mol HCl/liter; 0.5 ml; 30 min at 45°C) and the resulting quinoxaline derivatives purified by solid-phase extraction as given above.

Determination of Recovery and Yield

SPE recovery of amino acids was determined by comparing the amount of amino acids in the original serum sample and the NH₄OH eluate of the cation-exchange SPE. Prior to automatic amino acid analysis, ammonia was removed by addition of 0.02 ml NaOH (2 mol/liter) and evaporation to dryness.

Due to the vast excess of glycine in the incubation mixture, the extent of enzymatic conversion of L-amino acids was not feasible by automatic amino acid analysis. Therefore, incubation mixtures were spiked with trace amounts of ¹⁴C-labeled L-amino acids (1.6 kBq/ml). After enzymatic conversion and acidification, the label in 2-oxo[¹⁴C]acids was completely removed as ¹⁴CO₂ by treatment with H₂O₂ (30%, w/w, 0.1 ml) (24) and subsequent evaporation to dryness. The residue was dissolved in 0.2 ml water. Radioactivity in the remaining [¹⁴C]amino acids was determined by liquid scintillation counting. As controls, parallel samples were run in the absence of enzyme activity.

Final yields of quinoxaline derivatives were determined by high-performance liquid chromatographic analysis using pure quinoxaline derivatives for standardization (23).

Gas Chromatography of Quinoxalinol Derivatives

Prior to use, the quinoxaline derivative containing SPE eluates were evaporated to dryness under a stream of nitrogen.

Samples were then dissolved in 0.04 ml of a mixture of BSTFA and pyridine (1:1, v/v) and allowed to stand at ambient temperature for at least 15 min in order to form the O-TMS derivatives of the quinoxalins. The solution (0.2 to 0.5 μl) was injected into a splitless/split injector of a Model 3000 gas chromatograph (Varian, Darmstadt, Germany) equipped with a permabond OV-1 column (25 m, 0.32 mm i.d., 0.35 μm film thickness; from Macherey-Nagel, Düren, Germany) and directly connected to an INCON 50 mass spectrometer (Finnigan MAT, Bremen, Germany). Helium was used as carrier gas. Splitless time was 0.7 min, and split was 20:1. In general, the following temperature program was used: injector and transfer line to MS, 250°C; column oven 120°C initial and then increased at 10°C/min to 200°C and at 30°C/min to 280°C and held for 3 min.

Preparation of N-Trifluoroacetyl n-Propyl (TAP) Derivatives

Solutions of L-amino acids (50 nmol each) were evaporated to dryness. For propylation, 0.5 ml propanolic hydrochloric acid (2 mol/liter; prepared according to (25) from n-propanol and acetylchloride) was added. The mixture was heated at 60°C for 60 min in a sealed flask and there after evaporated to dryness under a stream of N₂. The residue was dissolved in 0.2 ml dichloromethane and the solvent removed under nitrogen. After addition of 50 μl dichloromethane and 100 μl trifluoroacetic anhydride and subsequent equilibration with N₂, the sample was allowed to stand in a closed vial at ambient temperature overnight. The mixture was then dissolved in 0.2 ml methanol and 0.2 ml trifluoroacetylating reagent (1:1, v/v) and allowed to stand at ambient temperature overnight. The mixture was then dissolved in 0.2 ml methanol and 0.2 ml trifluoroacetylating reagent (1:1, v/v) and allowed to stand at ambient temperature overnight.
was evaporated under a stream of nitrogen, 0.2 ml dichloromethane added, and evaporation repeated. Finally, the residue was dissolved in 0.2 ml dichloromethane and 0.5 μl of the solution analyzed by GC–MS using ammonia-CI and the program described above.

Mass Spectrometry

EI spectra were recorded at 70 eV ionization energy and an ion source temperature of 200°C. For CI, ammonia was used as reactant gas and the ion source was held at 70°C.

In the CI mode, the mass spectra of the O-TMS quinoxalinol derivatives showed only minor fragmentation. With the derivatives of natural L-al, L-glu, L-ile, L-leu, L-phe, and L-val (and derived 2-oxo acids), ion intensity was concentrated on the quasi-molecular ions [MH]+ appearing at m/z 233, 249, 275, 275, 309, and 263, respectively.

Ion intensity of the TAP derivatives of L-phe was concentrated on the quasi-molecular ion [MH]+ appearing at m/z 321.

Analysis of 13C-Enrichment

Ammonia-CI was generally applied. For determination of label enrichment in the O-TMS quinoxalinol derivative of L-[ring-13C6]phenylalanine, the ratio of ion intensity at m/z 315 to m/z 309 was measured by selected ion monitoring. The TAP derivative was analyzed at m/z 327 and m/z 321.

For measurement of 13C-label enrichment in serum samples after oral loading with L-[1-13C]valine, the ratio of ion intensity at m/z 264 to m/z 263 was monitored. For analysis of L-[1-13C]leucine and the corresponding 2-oxo acid, the ratio of ion intensity at m/z 276 to m/z 275 was determined.

Calculations

The mole percentage enrichment (MPE) of 13C-labeled branched-chain compounds in authentic serum samples was calculated from the ion abundance ratios R in postload samples and the natural abundance ratio R₀ as determined in the appropriate control samples. Ratios were normalized to an ion abundance of 1 at m/z 264 to m/z 263 was monitored. For analysis of L-[1-13C]leucine and the corresponding 2-oxo acid, the ratio of ion intensity at m/z 276 to m/z 275 was determined.

In general, results are presented as means ± SD (number of determinations in parentheses). Linear regression analysis (least-square method) was used for statistical evaluation.

RESULTS

Efficiency of Quinoxaline Synthesis

Specificity and yield of the enzymic-chemical procedure for conversion of serum L-amino acids into the corresponding quinoxaline derivatives were carefully checked for each of the L-amino acid dehydrogenases applied in this study. The compiled data are presented in Table 1.

The amino acids of interest were separated from 2-oxo acids by cation-exchange chromatography and recovered in >90% yield. With glutamate, recovery was somewhat lower due to necessity of prior removal of glutamine (and most other amino acids) by anion-exchange chromatography. When the latter procedure was examined with L-glutamine-enriched (2 mmol/liter) control sera, less than 0.5% of glutamine appeared in the glutamate fraction.

For enzyme treatment, incubation conditions were adjusted to achieve a reasonable yield of the desired 2-oxo acid and a minimum of interferences. As studied with 14C-labeled tracers, the time course of enzymatic conversion of L-amino acids was essentially hyperbolic. No significant amounts of interfering quinoxaline derivatives were detected by HPLC analysis. In incubations with L-phenylalanine dehydrogenase, however, minor amounts of the quinoxaline derivative of L-leucine accumulated (cf. Table 1). Furthermore, some phenylpyruvate was reaminated during prolonged incubation, most likely due to the, though slow, oxidative deamination of L-leucine and production of ammonia (cf. Fig. 1). Under our experimental conditions, efficiency of the enzymatic conversion was generally >70% (Table 1).

After derivatization of 2-oxo acids with o-phenylenediamine and purification by RP-SPE, variable yields of quinoxaline derivatives were obtained, ranging from about 3 to about 22 nmol/sample with the derivatives of L-glutamate and L-alanine, respectively (Table 1). The final yield primarily depended on the concentration of the L-amino acid in the serum sample and on the stability of the derived 2-oxo acid in the derivatization mixture. In all cases, however, the amount of quinoxaline derivative was more than sufficient for sensitive GC–MS analysis which required about 0.02 nmol.

GC–MS Analysis of [13C]Quinoxaline Derivatives

Applicability of the above enzymic-chemical procedure for measurement of isotope enrichment in individual L-amino acids was first tested using variably
TABLE 1
Recovery and Yield of Selective Enzymatic-Chemical Synthesis of Quinoxaline Derivatives of Amino Acids from Serum Samples

<table>
<thead>
<tr>
<th>Serum L-amino acid</th>
<th>SPE recovery (%)</th>
<th>Enzyme treatment</th>
<th>Enzymatic conversion (%)</th>
<th>Final yield of Qx derivatives (nmol/0.2 ml serum)</th>
<th>Rel yield of Qx derivatives (% of amino acid in serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>91.0 ± 7.2 (12)</td>
<td>Ala-DH</td>
<td>84.9 ± 9.0 (8)</td>
<td>21.7 ± 4.3 (8)</td>
<td>22.3 ± 6.1 (8)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>82.9 ± 5.5 (6)</td>
<td>Glu-DH</td>
<td>75.2 ± 8.4 (6)</td>
<td>3.1 ± 1.3 (6)</td>
<td>22.4 ± 6.3 (6)</td>
</tr>
<tr>
<td>L-isoleucine</td>
<td>93.3 ± 3.9 (12)</td>
<td>L-leu-DH</td>
<td>85.9 ± 4.1 (7)</td>
<td>4.0 ± 1.5 (73)</td>
<td>33.0 ± 9.0 (73)</td>
</tr>
<tr>
<td>Leucine</td>
<td>93.6 ± 3.1 (12)</td>
<td>Leu-DH</td>
<td>88.7 ± 1.5 (7)</td>
<td>10.5 ± 3.4 (73)</td>
<td>36.6 ± 8.6 (73)</td>
</tr>
<tr>
<td>Valine</td>
<td>93.2 ± 3.3 (12)</td>
<td>Val-DH</td>
<td>71.6 ± 7.2 (7)</td>
<td>8.5 ± 3.4 (73)</td>
<td>18.2 ± 6.0 (73)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>90.5 ± 3.0 (12)</td>
<td>Phe-DH</td>
<td>84.3 ± 4.9 (12)</td>
<td>3.9 ± 0.5 (12)</td>
<td>15.8 ± 0.9 (12)</td>
</tr>
<tr>
<td>L-leucine</td>
<td>93.6 ± 3.1 (12)</td>
<td>nd</td>
<td>0.09 ± 0.06 (12)</td>
<td>0.2 ± 0.1 (12)</td>
<td></td>
</tr>
</tbody>
</table>

Note. Amino acid fractions were isolated by deproteinization and ion-exchange chromatography (SPE recovery). Individual compounds were converted to the corresponding 2-oxo acids by treatment with L-amino acid dehydrogenases (DH) as indicated (enzymatic conversion). Quinoxaline (Qx) derivatives were formed by reaction with o-phenylenediamine and purified by reversed-phase chromatography (final yield). See Materials and Methods for details. Results are means ± SD with the number of determinations in parentheses; nd, not determined.

L-[ring-13C6]phenylalanine-enriched (0–99%, nine samples) control sera. The results in Fig. 2 show a good agreement of the calculated isotope enrichment and the data as obtained by GC–MS analysis of the O-TMS quinoxalinol derivatives. Noteworthy, a quite comparable correlation between predicted and measured 13C enrichment was obtained, when the N-trifluoroacetyl n-propyl derivatives of L-[13C]phenylalanine (samples in duplicate) were used for GC–MS analysis (linear regression analysis: y = 1.006x + 0.139; n = 18, R = 0.999; cf. legend to Fig. 2). Similar results were also achieved when O-TMS 2-butyl(3-methyl)-[13C]quinoxalinols were prepared from samples correspondingly enriched in L-[1-13C]leucine (data not shown).

The method was then applied successfully to serum analyses. Representative GC–MS traces of L-alanine and L-phenylalanine O-TMS derivatives as prepared from human serum samples are shown in Fig. 3. In

FIG. 1. Time course of the enzymatic conversion of L-phenylalanine in an amino acid fraction from human serum. Amino acids were isolated by deproteinization and cation-exchange chromatography and tracer doses of L-[1-14C]phenylalanine were added. Conversion of phe to the corresponding 2-oxo acid was achieved by treatment with L-phenylalanine dehydrogenase (glycine/NaOH, 0.2 mol/liter, pH 10.0; NAD+, 8 mmol/liter; 37°C; enzyme activity as indicated). The reaction was monitored by measuring the amount of H2O2-stable [14C]phe remaining in the incubation mixture. Controls were run in the absence of enzyme activity. For further details see Materials and Methods.

FIG. 2. Comparison of predicted and measured 13C-label enrichment (mole percentage excess, MPE) in L-[ring-13C6]phenylalanine from control sera as analyzed by GC–MS with the O-TMS quinoxalinol derivative. Ammonia-CI and selected ion monitoring (SIM) of the quasi-molecular ions [MH]+ and [MH + 6]+ appearing at m/z 309 and m/z 315 were used for measurement (see Fig. 3, bottom). Means of triplicate samples are shown (SD are included in the symbols). Dashed line, regression line (y = 0.999x + 0.153, n = 27, R = 0.999). For experimental details, see Materials and Methods.
our laboratory, the enzymic-chemical procedure is now routinely used for monitoring the time course of $^{13}$C enrichment in serum branched-chain compounds in stable isotope studies on the in vivo oxidation rates of branched-chain L-amino acids in healthy subjects and patients with maple syrup urine disease. Typical results from oral loading tests with L-$[^{13}C]$-labeled L-valine and L-leucine are presented in Fig. 4. The outcome of these studies and the medical implications will be reported elsewhere.

**DISCUSSION**

The present study concentrated on the examination of the use of L-amino acid dehydrogenases readily available from commercial sources for the selective enzymatic-chemical synthesis of quinoxaline derivatives from $^{13}$C- or $^2$H-labeled L-amino acids from physiological sources.

Irrespective of the GC–MS method used for measurement of label enrichment, L-amino acid fractions are generally purified by cation-exchange chromatography prior to derivatization. In the case of L-glutamate, rapid and careful separation from L-glutamine, normally present in vast excess in physiological samples, is required in order to avoid significant interferences from hydrolysis of the latter compound (25). We tried ion-exchange SPE columns for the purification of L-amino acids (primarily from interfering 2-oxo acids) and for the separation of glutamate. Recoveries (>90%) and separation efficiency (>99.5%) were comparable to published procedures (26–28).

Reportedly, the L-amino acid dehydrogenase activities applied exhibit more or less limited substrate specificities (29–31). Furthermore, the equilibrium constant for the oxidative deamination of amino acids is generally rather unfavorable (29,30). Nevertheless, under our incubation conditions, the L-amino acids in question, L-alanine, L-glutamate, and L-phenylalanine, and the group of branched-chain L-amino acids were converted to the derived 2-oxo acids by the belonging dehydrogenase activities with sufficient specificity and in reasonable yields (>70%). The procedure reported here for L-glutamate may also be applied to the synthesis of L-glutamine-derived quinoxaline derivative, if L-glutamine in the glutamate-free amino acid fraction is converted beforehand to L-glutamate by treatment with L-glutaminase (EC 3.5.1.2) (32).

Recovery of quinoxaline derivatives was variable and amounted to about 15 to 35% of the L-amino acid content in serum samples. With the L-amino acids studied, the most appreciable losses obviously occurred during derivatization of the sensitive 2-oxo acids in hydrochloric o-phenylenedia mine solution. Decomposition of some 2-oxo acid (presumably by decarboxylation (23,24)) prior to quinoxaline formation was inevitable, although rather mild conditions were applied for derivatization. The final yield of quinoxaline derivatives posed, however, no analytical problems. At least around 3 nmol was recovered from 0.2 ml serum, about 50-fold more than needed for sensitive GC–MS analysis.

For gas chromatographic separation of quinoxaline derivatives, silylation with BSTFA is the most widely used derivatization procedure. Using the branched-chain quinoxaline derivatives, we performed some additional GC–MS studies with underivatized quinox-
alines and with N,N-dimethylformamide dimethyl acetal as a derivatization reagent according to Fernandes et al. (33). When checked on the OV-1 column at our disposal, separation was not as excellent as with O-TMS derivatives. The underivatized quinoxalines exhibited more unfavorable gas chromatographic properties and the simultaneous formation of N- and O-methyl derivatives (33) rendered separation of methyl derivatives more difficult (results not shown).

Due to favorable gas chromatographic properties and negligible fragmentation in the CI mode (17–19), O-TMS derivatives were used throughout in the present study for GC–MS analysis of amino acid-derived quinoxalines.

Various applications were tested in order to examine the usefulness of the enzymatic chemical procedure for quantification of label enrichment in L-amino acids. With O-TMS quinoxalinol derivatives as prepared from solutions of variably enriched L-[ring-13C6]phenylalanine and L-[1-13C]leucine, a good correlation between predicted and actually measured label enrichment was obtained by GC–MS analysis using ammonia-CI and SIM of the quasi-molecular ions [MH]+ and [MH + n]+ (n = number of 13C-labeled carbons). Furthermore, measured 13C enrichments in solutions of L-[ring-12C]phenylalanine were practically identical when a commonly employed N-acyl alkyl ester (N-trifluoroacetyl n-propyl derivative) and the O-TMS quinoxalinol derivative were used for comparative GC–MS analysis.

Finally, the procedure was applied to monitor the dilution of 13C-label in serum branched-chain L-amino acids after oral loading with L-[1-13C]valine and L-[1-13C]leucine in in vivo metabolic studies on whole-body oxidation of branched-chain compounds in probands and patients with maple syrup urine disease. Due to the selective formation of branched-chain quinoxalinol derivatives, only few compounds had to be separated chromatographically and one common GC–MS program could be used for determination of label enrichment in a L-amino and the derived 2-oxo acid. Additionally, natural enrichment could be measured in each run, because all three branched-chain L-amino acids are converted to the quinoxaline derivatives and because no label exchange occurs between branched-chain L-amino acids in vivo.

This is especially useful in experiments with L-[1-13C]leucine, where an enriched and natural amount of 13C-label can be determined simultaneously by SIM at m/z 276 ([MH + 1]+) and m/z 275 ([MH]+) in the chromatographic peak of the O-TMS quinoxalinol derivative of L-leucine/4-methyl-2-oxopentanoate and L-isoleucine/3-methyl-2-oxopentanoate, respectively.

In conclusion, the present enzymic-chemical procedure appears to be well suited to sensitive determination of 13C or 2H enrichment in a number of L-amino acids. Expenditure for preparation of quinoxaline derivatives from L-amino acids is comparable to that of the most frequently used N-acylation O-alkylation procedures. However, the present method is more selective. N-O-Silyl derivatives of amino acids are rather unstable and multiple reaction products may be formed during derivatization (2,3). The O-TMS quinoxalinol derivatives are stable and exhibit most favorable GC–MS properties, especially when CI mode is applied. There is no need for two different GC–MS programs when 13C- and 2H-label enrichment must be measured in the L-amino as well as the corresponding 2-oxo acid.

In addition, use of 13C-labeled O-TMS quinoxalinol derivatives is not restricted to GC–MS analysis. According to our recent experience from in vivo experiments with labeled branched-chain L-amino acids, these compounds are also well suited to the determination of label enrichment by gas chromatography combustion isotope ratio–mass spectrometry ([34], Meier-Augenstein, W., and Schadewaldt, P., unpublished results).

In some cases, enzymatic conversion of L-amino to their corresponding 2-oxo acids and subsequent chemical decarboxylation (24) may also provide an applicable alternative to the ninhydrin method (35) which is now widely used for selective measurement of 13C enrichment in the CI-position of individual L-amino acids. Separation from interfering compounds is achieved by preparative GC in the latter and by enzyme specificity in the proposed procedure.

ACKNOWLEDGMENTS

We are indebted to Mr. L. Bohse for help with the amino acid analysis. This work was supported in part by Grant We 614/9-1 from the Deutsche Forschungsgemeinschaft.

REFERENCES