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Determination of *R*- and *S*-3-methyl-2-oxopentanoate enantiomers in human plasma: suitable method for label enrichment analysis[1]

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Abstract

A sensitive method for the determination of *S*- and *R*-3-methyl-2-oxopentanoate enantiomers (KMV, α-keto-β-methylvalerate) in physiological fluids suitable for isotope enrichment analysis is described: after extraction with acid, 2-oxo acids are separated from interfering amino acids by cation-exchange chromatography. Reductive amination of the branched-chain 2-oxo acids by use of L-leucine dehydrogenase yields the corresponding L-amino acids. L-Isoleucine and L-alloisoleucine which are formed from *S*- and *R*-3-methyl-2-oxopentanoate, respectively, are then quantified by amino acid analysis. The method was used for determination of the *R*-/*S*-3-methyl-2-oxopentanoate ratio in plasma of healthy subjects and patients with diabetes mellitus and maple syrup urine disease. Applicability for gas chromatographic–mass spectrometric analysis of ^{13}C-label enrichment in plasma *S*-3-methyl-2-oxopentanoate is demonstrated.

Keywords: Enantiomer separation; 3-Methyl-2-oxopentanoate

1. Introduction

The essential branched-chain L-amino acids L-leucine, L-valine, and L-isoleucine (2*S*,3*S*) and their transamination products 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and *S*-3-methyl-2-oxopentanoate are normal constituents of human plasma. Increased concentrations are regularly found in patients with maple syrup urine disease. The presence of appreciable amounts of L-alloisoleucine (2*S*,3*R*) and the corresponding 2-oxo acid, *R*-3-methyl-2-oxopentanoate, is pathognomonic for this metabolic disorder (cf. Fig. 1), where the catabolism of branched-chain compounds is impaired by an inherited deficiency of branched-chain 2-oxo acid dehydrogenase complex activity (EC 1.2.4.4) (see [1,2] for review).

L-Alloisoleucine has been shown to be formed from L-isoleucine in vivo. However, the underlying mechanism is still unclear [3]. Originally it had been suggested that keto-enol tautomerization of L-isoleucine derived *S*-3-methyl-2-oxopentanoate leads to the formation of the enantiomeric *R*-2-oxo acid. L-Alloisoleucine is then formed by subsequent retransamination, which is catalyzed by branched-chain L-amino acid aminotransferase (EC 2.6.1.42) [4]. This mechanism has recently been questioned,

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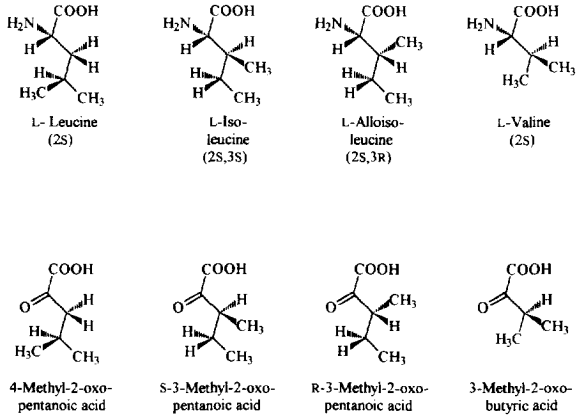

Fig. 1. Branched-chain L-amino acids and their corresponding 2-oxo acids occurring in human plasma.

however, in consideration of the very low rates of 2-oxo acid racemization under in vivo conditions. Epimerization of L-isoleucine after binding to the pyridoxal-moiety of the aminotransferase and direct release of L-alloisoleucine has therefore been envisaged as a possible metabolic pathway [5].

For study of the interrelation of L-isoleucine and L-alloisoleucine in vivo, sensitive analysis of the L-amino and their derived 2-oxo acids is necessary. Numerous appropriate methods for the measurement of the diastereomeric L-amino acids are available. In contrast, only two procedures for analysis of the enantiomeric 2-oxo acids have been published. For the polarimetric assay described by Weinberg and Walser [6] large sample volumes are needed. Therefore, it is not applicable for kinetic in vivo studies in humans. The second method is based on amino acid analysis before and after reductive amination of the 2-oxo acids by treatment with L-leucine dehydrogenase (EC 1.4.1.9) [7]. This previous method is of sufficient accuracy when the 2-oxo acid/L-amino acid ratio is rather high as is the case e.g., in samples from maple syrup urine disease patients. In the plasma of healthy subjects, however, reliable analysis of *S*- and *R*-3-methyl-2-oxo acids cannot be performed. Furthermore, the method is not applicable for the determination of stable isotope enrichment in the individual 2-oxo acid enantiomers which may be necessary when ^{13}C- or ^{2}H-labelled L-isoleucine or L-alloisoleucine are to be used e.g., for the study of in vivo metabolic fluxes in man [8].

We have now established a method which overcomes these limitations. It allows sensitive quantification of low *R*- in the presence of high *S*-3-methyl-2-oxopentanoate concentrations. It was used for the study of the relation of branched-chain L-amino and 2-oxo acids in healthy subjects and in patients. Applicability for the estimation of stable isotope enrichment in 3-methyl-2-oxopentanoate enantiomers by gas chromatography–mass spectrometry is demonstrated.

2. Experimental

2.1. Chemicals

Unless otherwise noted all chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (Munich, Germany) in the highest available purity. Racemic *R,S*-3-methyl-2-oxopentanoate, calcium salt, was a gift from Degussa (Frankfurt, Germany). Coenzymes, catalase (EC 1.11.1.6, from bovine liver) and L-amino acid oxidase (EC 1.4.3.2 from *Crotalus durissus*) were from Boehringer (Mannheim, Germany), L-leucine dehydrogenase (EC 1.4.1.9; from *Bacillus* sp.) from Sigma. L-[1-^{13}C]Isoleucine (1-^{13}C, 99%) was obtained from Promochem (Wesel, Germany). As checked by amino acid analysis, the preparation was free from L-alloisoleucine. It was used to prepare pure *S*-3-methyl-2-oxo[1-^{13}C]pentanoate by treatment with L-amino acid oxidase as described by Rüdiger et al. [9].

2.2. Separation of amino and 2-oxo acids from plasma

In order to allow reliable determinations in samples with low 2-oxo acid concentrations the following procedure was used: as internal standards, 0.05 ml of 2-oxohexanoate (0.4 mmol/l) and DL-norleucine (2 mmol/l) were added to 1 ml plasma sample. Plasma from patients with maple syrup urine disease were diluted (1:5, v/v) with NaCl (0.154 mol/l) prior to use. The solution was mixed with 0.06 ml of 5-sulfosalicylic acid (60%, w/v). After centrifugation (10 000 *g*, 4°C, 10 min), 0.75 ml of the supernatant was applied onto a Dowex 50 WX8 column (100–200 mesh; 19×6 mm in disposable

SPE columns from Baker, Groß-Gerau, Germany) equilibrated with 5-sulfosalicylic acid (3%, w/v). The column was washed with 0.5 ml 5-sulfosalicylic acid (3%, w/v). The first 0.5 ml of the effluent were discarded. The remaining fraction (0.75 ml) contained the 2-oxo acids and was free from amino acids. It was used for enzymatic treatment as described below.

For subsequent isolation of amino acids, the column was washed with 1 ml H_2O and the amino acids then eluted with 1 ml NH_4OH (2.5 mol/l). The last 0.75 ml of the alkaline eluate was collected and evaporated to dryness under a stream of nitrogen. The residue was used for automatic amino acid or GC–MS analysis.

2.3. Enzymatic amination of 2-oxo acids

Prior to analysis, the corresponding L-amino acids were formed from the 2-oxo acids according to the equation:

$$2-\text{Oxo acid} + \text{NADH} + \text{NH}_4^+ \xrightarrow{\text{L-Leucine Dehydrogenase}} \text{L-Amino acid} + \text{NAD}^+ + \text{H}_2\text{O}$$

For conversion, the 2-oxo acid fraction (0.75 ml) was mixed with NH_4Cl/NH_4OH-buffer (0.15 ml, 5 mmol/l, pH 8.35), NH_4OH solution (0.1 ml, 2.5 mol/l), and NADH (0.06 ml, 100 mmol/l). L-Leucine dehydrogenase (0.3 U in 0.25 ml) was added and the mixture then incubated at 37°C for 2 h. After addition of 5-sulfosalicylic acid (1 ml, 20%, w/v), the L-amino acids formed were purified and concentrated by Dowex 50 WX8 chromatography as described above.

2.4. Amino and 2-oxo acid analysis

For amino acid analysis, dry residues were resolved in an appropriate volume of 5-sulfosalicylic acid (10%, w/v) and applied onto a DC 4A column (160×6 mm) of an automatic amino acid analyser (Biotronik LC 6000; Eppendorf Biotronik, Maintal, Germany). Isocratic conditions were used for elution (lithium-citrate buffer, 0.35 mol Li^+/l, pH 3.5, 33 ml/h at 52°C). Ninhydrin was used for detection and quantification performed on hand of the internal standard (norleucine) and external calibration runs.

The procedure for branched-chain 2-oxo acid analysis has been previously described in detail [10]. In short, quinoxaline derivatives of the 2-oxo acids in deproteinized samples were obtained by reaction with *o*-phenylenediamine, purified by solid-phase extraction on a 1-ml C_{18} column (from Baker) and quantified using C_{18} reversed-phase high-performance liquid chromatography and fluorescent detection. 2-Oxohexanoate was used for internal standardization, and appropriate blanks and standard solutions were run in parallel.

2.5. Determination of ^{13}C-label enrichment

Samples were obtained by spiking plasma from a patient with maple syrup urine disease with pure *S*-3-methyl-2-oxo[1-^{13}C]pentanoate (1-^{13}C, 99%). For selective measurement of ^{13}C-label in *S*- and *R*-3-methyl-2-oxopentanoate, plasma branched-chain 2-oxo acids were enzymatically converted to the corresponding L-amino acids as detailed above. After purification and evaporation to dryness, the N-acetyl-O-isopropyl derivatives were prepared: 0.5 ml isopropanolic hydrochloric acid (2 mol/l; prepared according to [11]) was added and the mixture heated at 60°C for 60 min in a sealed flask. After evaporation to dryness under a stream of nitrogen, 0.2 ml dichloromethane was added and evaporation repeated. Dichloromethane (0.05 ml) and 0.1 ml acetic acid anhydride were added, the sample equilibrated with N_2, and then allowed to stand in a closed vial at ambient temperature overnight. After evaporation, 0.2 ml dichloromethane was added and evaporation repeated. Finally, the residue was dissolved in 0.2 ml dichloromethane and 0.1–0.5 μl analyzed by GC–MS.

GC–MS analysis was performed using a Varian 300 gas chromatograph (Varian, Darmstadt, Germany) equipped with a splitless/split injector, a permabond OV-1 column (100% dimethylpolysiloxane, 25 m×0.32 mm I.D., 0.35 μm film thickness; from Macherey and Nagel, Düren, Germany) and directly connected to an INCOS 50 mass spectrometer (Finnigan MAT, Bremen, Germany). Helium was used as carrier gas. Splitless time was 0.7 min, and split was 20:1. Injector and transfer line to MS were

held at 250°C. The column oven was 90°C initial and then increased at 2°C/min to 130°C, at 30°C/min to 280°C and held for 3 min. Positive chemical ionisation was applied, ammonia was used as reactant gas. The ion source was held at 70°C.

For determination of label enrichment in the N-acetyl-O-isopropylesters of L-isoleucine and L-alloisoleucine derived from spiked *S*-3-methyl-2-oxo-[1-^{13}C]pentanoate and natural labelled *R*-3-methyl-2-oxopentanoate, respectively, ion intensities of the quasi molecular ions $[MNH_4]^-$ and $[MNH_4+1]^+$ (appearing at *m/z* 233 and *m/z* 234, respectively) were measured. The ratio of ion intensities *m/z* 234 to *m/z* 233 in the chromatographic peaks of the L-alloisoleucine ($=R_0$) and L-isoleucine derivatives ($=R$) were used for the determination of natural abundance and label enrichment, respectively. Ratios were normalized to an ion abundance of 1 of the respective quasi-molecular ion $[MNH_4]^+$.

2.6. Calculations and statistics

The mole percent enrichment (MPE) of ^{13}C-labelled compounds was calculated [12] from the natural abundance ratio ($=R_0$) and enriched sample ratio ($=R$) as $MPE=(R-R_0)/\{1+(R-R_0)\}\times 100$.

In general, results are presented as means±S.D. with the number of determinations in parentheses. Correlations were examined by linear regression analysis (least-squares method).

3. Results

3.1. Specificity, precision and yield

We carefully checked the individual steps of the procedure. Under the conditions applied, plasma branched-chain 2-oxo acids were completely freed from interfering L-amino acids by cation-exchange chromatography. Noteworthy, a partial fractionation of branched-chain 2-oxo acids and their straight chain analogues occurred (cf. Fig. 2). In general, 2-oxohexanoate was applied for standardization and care was taken to collect the physiological and standard 2-oxo acids in equal amounts. As determined by HPLC analysis using 2-oxopentanoate for internal standardization, the yields of *R,S*-3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate, 2-oxohexanoate, and 3-methyl-2-oxobutyrate at this step were 85.2±3.0, 86.8±3.1, 85.6±1.9, and 83.5±6.5%, respectively ($n=27$).

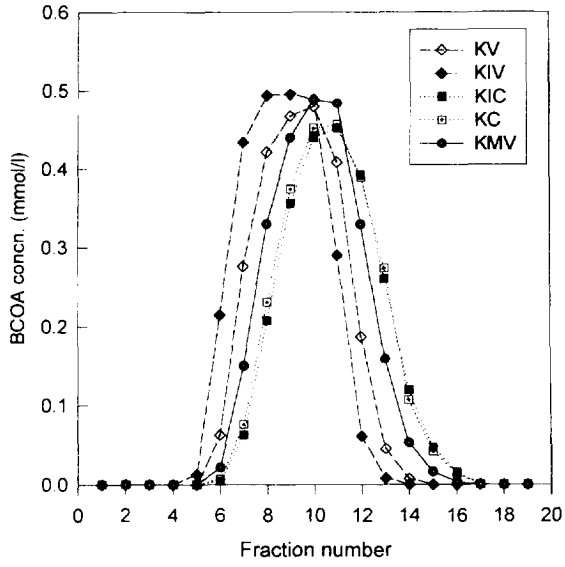

Fig. 2. Partial fractionation of branched-chain 2-oxo acids and straight-chain analogues on Dowex 50 WX8. 2-Oxo acids (0.5 mmol/l) in 5-sulfosalicylic acid (3% w/v) were applied onto the column [19×6 mm; equilibrated with 3% (w/v) 5-sulfosalicylic acid] and washed with 5-sulfosalicylic acid (3%, w/v). Fractions (0.1 ml) of the effluent were collected and analyzed for 2-oxo acid concentrations by HPLC of the quinoxaline derivatives (cf. Section 2). Abbreviations: KV=2-oxopentanoate; KIV=3-methyl-2-oxobutyrate; KIC=4-methyl-2-oxopentanoate; KC=2-oxohexanoate, KMV=*R,S*-3-methyl-2-oxopentanoate.

The subsequent enzyme catalysed reductive amination of the 2-oxo acids to form the corresponding L-amino acids was essentially complete. No measurable amounts of 2-oxo acids could be detected at the end of incubation. As examined by amino acid analysis ($n=7$; cf. Fig. 3) using L-phenylalanine as an internal standard, L-isoleucine (89.4±4.7%), L-alloisoleucine (88.0±3.5%), L-leucine (88.3±3.0%), L-norleucine (90.0±1.8%) and L-valine (87.4±4.2%) were recovered in comparable amounts after purification and concentration by Dowex 50 WX8 chromatography.

When related to the 2-oxo acid content in the original plasma samples, the final yields of the L-amino acids derived from *S*- plus *R*-3-methyl-2-

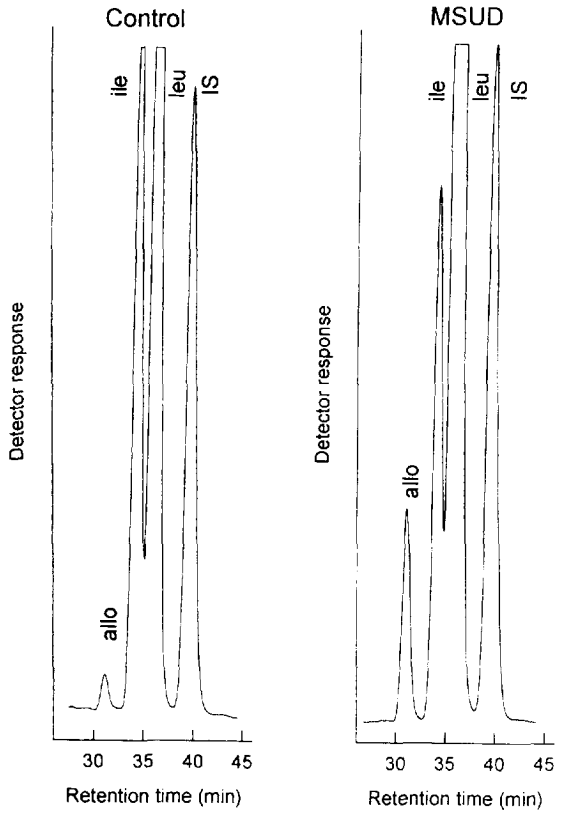

Fig. 3. Chromatographic separation of branched-chain L-amino acids derived from *S*- and *R*-3-methyl-2-oxopentanoate in plasma of a healthy subject and a patient with maple syrup urine disease (MSUD). 2-Oxo acids were isolated from plasma, enzymatically converted to the L-amino acids, and analyzed by cation exchange chromatography using ninhydrin detection as detailed under Section 2. MSUD plasma was diluted 1:5 prior to use. Abbreviations: allo = L-alloisoleucine; ile = L-isoleucine; leu = L-leucine; IS = internal standard (L-norleucine).

oxopentanoate, 4-methyl-2-oxopentanoate, 2-oxohexanoate, and 3-methyl-2-oxobutyrate amounted to 47.7±3.6, 49.4±2.7, 48.9±3.4 and 48.7±3.6% ($n = 10$), respectively.

Reproducibility was examined in the low as well as the high concentration range using pool plasma which was spiked with branched-chain 2-oxo acids appropriately. Under strictly controlled conditions, the method proved to be highly reproducible as documented by the data in Table 1.

When the ratio of *S*-/*R*-3-methyl-2-oxopentanoate was varied over a wide range by spiking human plasma with increasing amounts of racemic *R,S*-3-methyl-2-oxopentanoate, a good correlation between expected and actually measured ratios was obtained (Fig. 4).

Finally, total *R,S*-3-methyl-2-oxopentanoate content in plasma as measured by HPLC analysis of the quinoxaline derivatives was compared with the data which were obtained by amino acid analysis (sum of L-isoleucine and L-alloisoleucine) after enzymatic amination. 2-Oxohexanoate was used throughout for internal standardization. The results in Fig. 5 show a reasonable correlation between the two methods. As related to HPLC analysis, the mean recovery of *R,S*-3-methyl-2-oxopentanoate derived L-amino acids in a total of 41 different samples amounted to 104±8%.

3.2. S- and R-3-Methyl-2-oxopentanoate in human plasma

The method was applied for the study of the relation of L-isoleucine, L-alloisoleucine and their corresponding 2-oxo acids in the plasma of healthy subjects and of patients with diabetes mellitus and maple syrup urine disease.

The compiled data in Table 2 show that *R*-3-methyl-2-oxopentanoate and L-alloisoleucine were feasible in plasma of healthy subjects and patients with diabetes mellitus although at rather low concentrations. In controls ($n = 15$), the *R*-enantiomer amounted to 2.7±0.8% of total 3-methyl-2-oxopentanoate in plasma. The ratio of L-alloisoleucine/L-isoleucine (0.028±0.009) and *R*-/*S*-3-methyl-2-oxopentanoate (0.031±0.005) as well as the apparent in vivo transamination equilibria, i.e., the ratios of *S*-3-methyl-2-oxopentanoate/L-isoleucine (0.33±0.07) and *R*-3-methyl-2-oxopentanoat/L-alloisoleucine (0.38±0.09) were comparable. Quite similar observations were made in the plasma of diabetic patients (data not shown).

As expected, a wide range of metabolite concentrations was found in plasma samples ($n = 21$) from six maple syrup urine disease patients (cf. Table 2). The *R*-enantiomer represented 30±5% of total 3-methyl-2-oxopentanoate, the ratio of *R*-/*S*-3-methyl-2-oxopentanoate was 0.44±0.10. Somewhat unexpected was the finding of an apparent strong linear correlation between the plasma concentrations

Table 1
Reproducibility of branched-chain 2-oxo acid determination in human plasma

Branched-chain 2-oxo acid	Coefficient of variation (%)			
	Within-run ($n=10$)		Between-run ($n=9$)	
	Low concentration range	High concentration range	Low concentration range	High concentration range
S-3-Methyl-2-oxopentanoate	2.9 (24.5±0.7)	1.9 (262±5)	2.1 (23.9±0.5)	2.0 (254±5)
R-3-Methyl-2-oxopentanoate	7.5 (0.93±0.07)	2.1 (243±5)	6.2 (0.98±0.06)	1.8 (240±4)
4-Methyl-2-oxopentanoate	3.6 (34.9±1.3)	1.9 (249±5)	1.6 (34.5±0.5)	2.1 (237±5)
3-Methyl-2-oxobutyrate	4.6 (16.2±0.7)	2.0 (249±5)	3.4 (16.8±0.6)	3.4 (238±8)

2-Oxo acids were enzymatically aminated and the resulting L-amino acids measured by amino acid analysis as detailed in Section 2. Fresh pool plasma was used in the low concentration range. For preparation of a high concentration sample, an essentially 2-oxo acid free plasma was chosen and spiked with 0.25 mmol/l of each branched-chain 2-oxo and L-amino acid. The concentrations of L-isoleucine, L-alloisoleucine, L-leucine, and L-valine in the low (high) sample amounted to 73 (343), 1.6 (240), 158 (428), and 252 (502) μmol/l, respectively. For convenience, measured 2-oxo acid concentrations (mean ±S.D., in μmol/l) are included in parentheses.

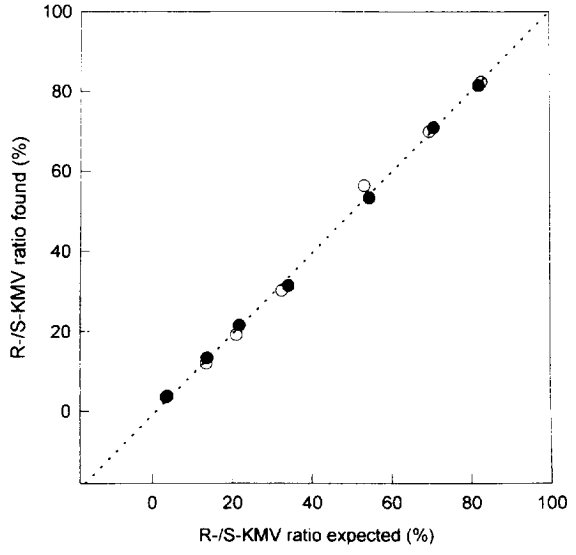

Fig. 4. Analysis of the relation of *S*- and *R*-3-methyl-2-oxopentanoate (KMV) in human plasma. The ratio of the enantiomers was varied by spiking two fresh plasma samples with racemic *R,S*-3-methyl-2-oxopentanoate (0, 5, 10, 20, 50, 100 and 200 μmol/l). Calculation of the expected ratio was based on the *R*-/*S*-content of the original samples and the added amounts of racemate. 2-Oxo acids were determined by amino acid analysis after enzymatic conversion to the respective L-amino acids (cf. Section 2 for details). Dotted line = regression line ($y=1.01x-0.99$, $r=0.999$).

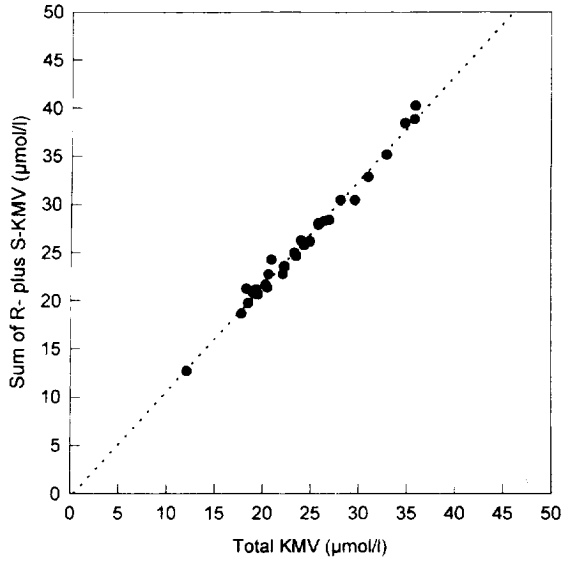

Fig. 5. Comparison of *R,S*-3-methyl-2-oxopentanoate (KMV) determinations in plasma samples of healthy subjects and diabetic patients. Total 2-oxo acid concentration was estimated by RP-HPLC of the quinoxaline derivative and correlated to the sum of *R*- plus *S*-KMV concentrations which were measured by amino acid analysis after enzymatic conversion to the corresponding amino acids L-isoleucine and L-alloisoleucine (cf. Section 2). Dotted line = regression line ($y=1.09x-0.35$, $r=0.993$, $n=30$).

Table 2
Concentration of *S*- and *R*-3-methyl-2-oxopentanoate and the corresponding amino acids in human plasma

Branched-chain compound	Plasma concentration (μmol/l)		
	Controls ($n=15$)	Diabetics ($n=15$)	MSUD patients[a] ($n=21$)
S-3-Methyl-2-oxopentanoate	24.0 ± 6.2	26.6 ± 5.6	59.7±49.9 Range: 17–208
L-Isoleucine	72.8 ±10.0	81.5 ±12.8	101.0±7 1.2 Range: 32–312
R-3-Methyl-2-oxopentanoate	0.76± 0.19	0.92± 0.33	25.3±19.5 Range: 7– 78
L-Alloisoleucine	2.00±0.55	1.75± 0.53	71.9±29.5 Range: 26–136

Fresh EDTA–plasma was used. 2-Oxo acids were isolated, enzymatically converted to the corresponding L-amino acids, and quantifications performed by amino acid analysis (cf. Section 2). Results are means ±S.D. Number of probands/patients are given in parentheses.
[a] Refers to the number of independent samples obtained from six different patients with maple syrup urine disease (MSUD).

of the two 2-oxo acids (cf. Fig. 6) when compared to the rather variable L-alloisoleucine/L-isoleucine ratio which amounted to 0.81±0.28.

3.3. Label enrichment analysis

We finally tested applicability of the method for measurement of stable isotope enrichment. So far, appropriate samples from in vivo stable isotope studies are not available. Therefore, plasma from a patient with maple syrup urine disease with increased branched-chain L-amino and 2-oxo acids was spiked with an appropriate amount of pure *S*-3-methyl-2-oxo[1-^{13}C]pentanoate to give a defined label enrichment. The label in the *S*- and *R*-enantiomorph derived L-amino acids was then determined by gas chromatography–mass spectrometry.

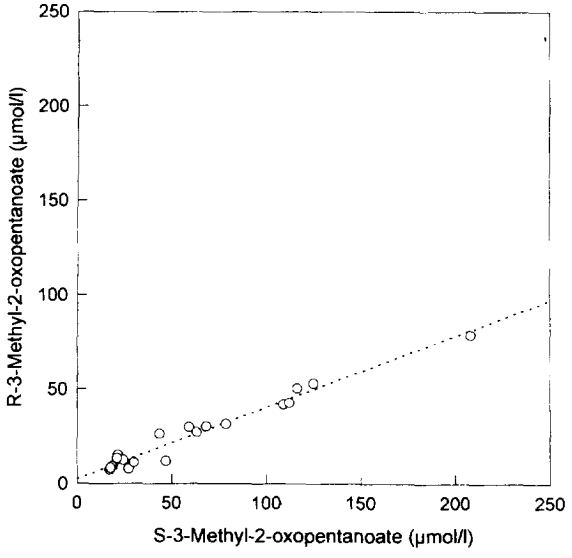

Fig. 6. Correlation of *S*- and *R*-3-methyl-2-oxopentanoate concentration in plasma of maple syrup urine disease patients. Samples were obtained from six different patients. Concentrations were estimated by amino acid analysis after enzymatic conversion of the 2-oxo acids to the corresponding L-amino acids (cf. Section 2). Dotted line = regression line ($y=2.54x+0.38$, $r=0.982$, $n=21$).

On the specific capillary column connected to the mass spectrometer at our disposal, the N-acetyl-O-isopropyl derivatives of L-isoleucine (corresponding to *S*-3-methyl-2-oxopentanoate) and L-norleucine (corresponding to 2-oxohexanoate, I.S.) were completely separated, while the derivatives of L-leucine (corresponding to 4-methyl-2-oxopentanoate) and L-alloisoleucine (corresponding to *R*-3-methyl-2-oxopentanoate) were not fully resolved. However, baseline separation of the N-acetyl as well as the N-trifluoroacetyl-O-isopropyl-but not the O-TMS-derivatives was readily achieved (H.-W. Hammen and P. Schadewaldt, unpublished) under quite similar conditions on a separate gas-chromatograph equipped with a DB-1 column (100% dimethylpolysiloxane, 30 m×0.25 mm I.D., 0.25 μm film thickness). Thus, a column of tested performance is to be used when perfect resolution of all components is desired.

In Fig. 7, mass spectrometric results of a 10% enriched sample are shown. Based on the ratio of ion intensities at *m/z* 234 to *m/z* 233 in the L-isoleucine

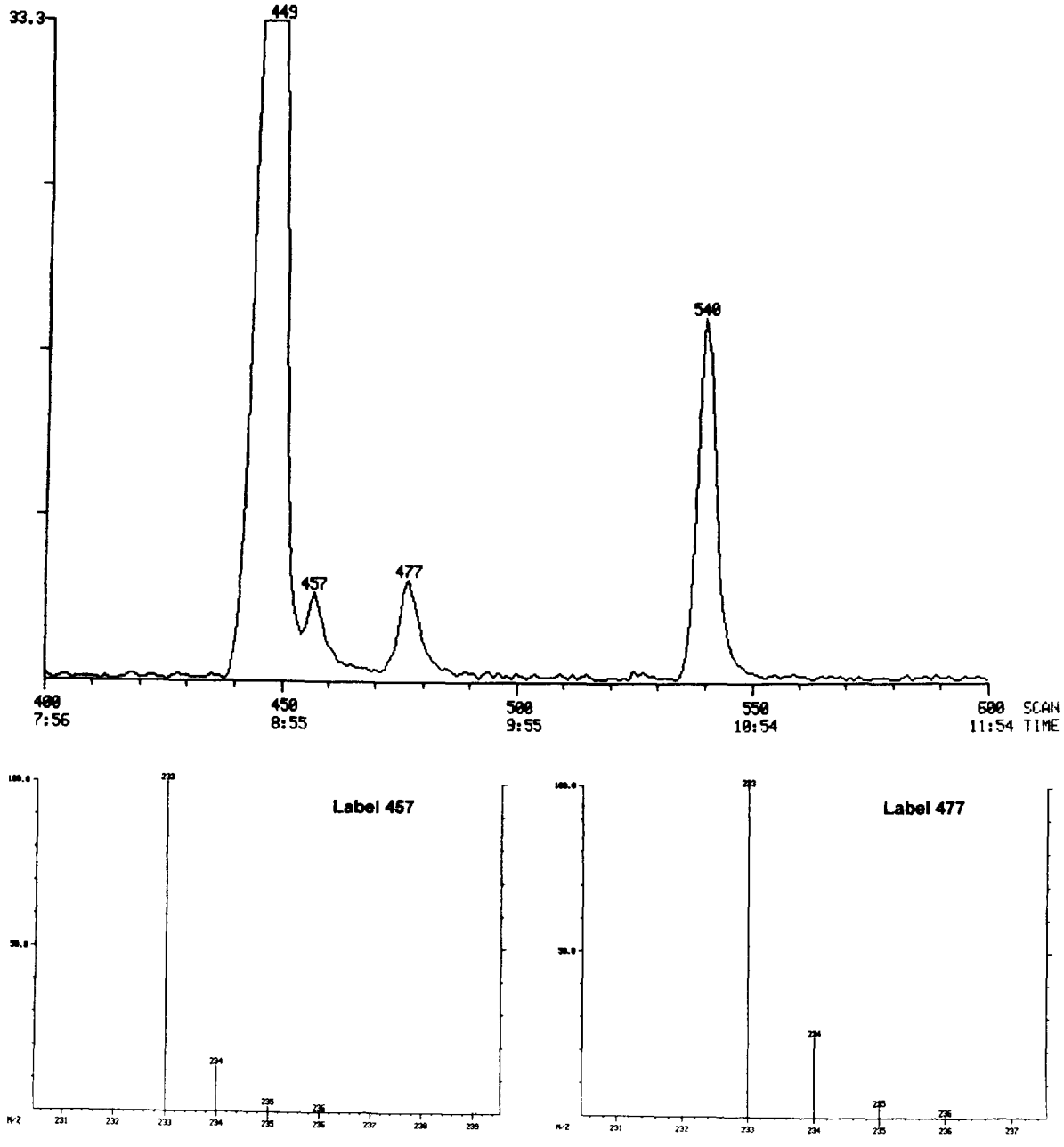

Fig. 7. GC–MS analysis of ^{13}C-label enrichment in *R,S*-3-methyl-2-oxopentanoate in plasma. (Top) Separation of the N-acetyl-O-isopropyl L-amino acid derivatives prepared from 4-methyl-2-oxopentanoate (label 449), *R*-3-methyl-2-oxopentanoate (label 457), *S*-3-methyl-2-oxopentanoate (label 477) and 2-oxohexanoate (label 540; internal standard). Plasma from a patient with maple syrup urine disease was spiked with pure *S*-3-methyl-2-oxo[1-^{13}C]pentanoate to give a 1-^{13}C-label enrichment of 10% and used for analysis. Ammonia-CI and expanded scan mode (210–260 a.m.u.) was applied. (Bottom) Short scan CI-mass spectra of the quasi-molecular ions $[MNH_4]^+$ and $[MNH_4+1]^+$. Unlabelled and 1-^{13}C-labelled derivatives of *S*- and *R*-3-methyl-2-oxopentanoate appear at *m/z* 233 and at *m/z* 234, respectively.

(ratio: 0.246) and L-alloisoleucine (ratio: 0.139) chromatographic peaks, a *S*-3-methyl-2-oxopentanoate ^{13}C-enrichment of 9.7% was calculated. This is in rather good agreement with the theoretical value. Equivalent results were obtained with a 5% enriched plasma sample (data not shown).

4. Discussion

The results of this study show that *R*-3-methyl-2-oxopentanoate plasma levels can be reliably measured in the presence of a large excess of the *S*-enantiomer when the 2-oxo acids are separated from interfering amino acids by cation-exchange chromatography prior to enzymatic formation and analysis of the corresponding L-amino acids. Amino acid analysis was facilitated and accelerated because only a few components had to be separated. Sensitivity of the procedure can be readily increased by an order of magnitude by using post-column derivatization with *o*-phthaldialdehyd and fluorimetric detection instead of the conventional ninhydrin method or by employing modern reversed-phase HPLC techniques if desired. Under our experimental conditions, the procedure proved to be rather sensitive and highly reproducible at low normal metabolite levels as well as in the high concentration range occurring in maple syrup urine disease.

Using this method, we were able to quantify for the first time the relation of *S*- and *R*-3-methyl-2-oxopentanoate in subjects other than maple syrup urine disease patients. It is evident from the data that the *R*-enantiomer accounts for only a minor portion (<3%) of the total 3-methyl-2-oxopentanoate in normal plasma. Thus, the relation of the 2-oxo acids was similar to the relation of the respective plasma L-amino acids, L-isoleucine (2*S*,3*S*) and L-alloisoleucine (2*S*,3*R*), indicating comparable in vivo transamination equilibria of the two L-amino/2-oxo acid pairs. In maple syrup urine disease patients, plasma levels of L-amino and 2-oxo acids are significantly elevated due to the impaired branched-chain 2-oxo acid decarboxylation step. The present results on increased concentrations and the ratio of *R*- and *S*-3-methyl-2-oxopentanoate are in agreement with previous data [7] and are possibly explained by the trap function of L-alloisoleucine metabolism in this disorder [3].

It is interesting to note in this context that low concentrations of *R*,*S*-3-methyl-2-oxopentanoate derived 2-hydroxy acids have also been found in human plasma. Presumably, these metabolites are formed by action of L-lactate dehydrogenase (EC 1.1.1.27). According to published data, total *R*,*S*-2-hydroxy-3-methylpentanoate plasma levels are about 0.4 μmol/l in normal subjects, the *R*-enantiomer accounting for about 30% thereof [5]. Therefore, the *R*-form appears to be considerably enriched when related to the enantiomeric composition of the 2-oxo acid precursor pool where the *S*-form accounts for more than 95%. Whether this difference may depend on different substrate properties of the *R*- and *S*-2-oxo acid for the dehydrogenase step or rather reflect differences in the renal clearance rates of the *S*- and *R*-form [5] is unknown at present and remains to be investigated.

The method has also been shown to be applicable for the measurement of ^{13}C-isotope enrichment individually in *S*- and *R*-3-methyl-2-oxopentanoate by gas chromatography–mass spectrometry. This may be useful for stable isotope studies in man e.g., when experiments are designed towards an answer to the long standing question on the mechanism initiating the formation of the 3-*R* series of L-isoleucine metabolites in vivo.

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