ELSEVIER

Journal of Chromatography B, 738 (2000) 99–105

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of isotopic enrichments of [1-^{13}C]homocysteine, [1-^{13}C]methionine and [$^{2}H_3$-methyl-1-^{13}C]methionine in human plasma by gas chromatography–negative chemical ionization mass spectrometry

W. Kulik*, R.M. Kok, K. de Meer, C. Jakobs

Department of Clinical Chemistry, University Hospital Vrije Universiteit, PO Box 7057, De Boelelaan 1117, NL-1081 HV Amsterdam, The Netherlands

Received 25 August 1999; received in revised form 18 October 1999; accepted 27 October 1999

Abstract

We describe a reliable method for the simultaneous determination of isotopic enrichments of [1-^{13}C]homocysteine, [1-^{13}C]methionine and [$^{2}H_3$-methyl-1-^{13}C]methionine in human plasma. Accurate [1-^{13}C]homocysteine calibration standards were prepared by chemical conversion via thiolactonisation of [1-^{13}C]methionine standards. Based upon anion-exchange chromatography, (di)acetyl-3,5-bis–trifluoromethylbenzyl derivatives, preparation of accurate calibration curves and gas chromatography–negative chemical ionization mass spectrometry, isotopic enrichments in human plasma could be determined with TTR (%) $<\pm 0.2\%$ ($N=3$) for [1-^{13}C]homocysteine (enrichment range 0–8%), [1-^{13}C]methionine (enrichment range 0–3%) and [$^{2}H_3$-methyl-1-^{13}C]methionine (enrichment range 0–12%). The method was applied in a [$^{2}H_3$-methyl-1-^{13}C]methionine tracer infusion study in a biological model.

Keywords: Homocysteine; Methionine

1. Introduction

The essential amino acid methionine can be transmethylated, through the intermediates *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH), into homocysteine. In mammals the latter amino acid follows only two pathways. Homocysteine can be degraded through the transsulfuration, leading to the formation of e.g., cystathionine, cysteine, glutathione and ultimately α-ketoglutaric acid and CO_2, or homocysteine can be remethylated to methionine [1].

The use of methionine tracers labelled with [methyl-$^{2}H_3$] or [1-^{13}C], respectively, [2] or methionine with both labels [3–6] makes it possible to study the in vivo dynamics of the methionine cycle quantitatively, using mass spectrometric analyses of the isotopic labelling of methionine in plasma (as the *N*-acetyl, *n*-propyl derivative) and $^{13}CO_2$ enrichment in expired air using isotope ratio mass spectrometry (IRMS). Using this methodology it is possible to study the fate of homocysteine, a compound of

*Corresponding author. Tel.: +31-20-444-2881; fax: +31-20-444-0305.

E-mail address: w.kulik@azvu.nl (W. Kulik)

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00504-6

recent and increasing interest in the field of cardiovascular disease (reviewed in [7–12]).

For the determination of plasma concentrations of homocysteine and its related compounds in plasma there already exist several assays for analytical detection based on immunoassay, HPLC (fluorescence/electrochemical detection), GC or GC–MS (see [13–17]). Until now it seems, however, not to be possible to use these methods interchangeably [18]. As a basis for stable isotope studies on homocysteine kinetics, we describe an improved method for the combined analysis of the isotopic labelling in both homocysteine and methionine in plasma with electron capture chemical ionization (ECCI) mass spectrometry. We discuss precision, accuracy and linearity of this method as well as the preparation of accurate [1-^{13}C]homocysteine standards by chemical conversion of chemically pure [1-^{13}C]methionine/methionine. The applicability of the method is illustrated by a [$^{2}H_3$-methyl-1-^{13}C]methionine tracer infusion in a biological model (healthy human).

2. Experimental

2.1. In vivo experiments

Healthy volunteers were studied in the fasting state as previously described [19]. During the infusion, a tracer amount of $NaH^{13}CO_3$ was given as an intravenous bolus at $t=0$ (to prime the bicarbonate pool), and immediately thereafter an intravenous primed continuous infusion of [$^{2}H_3$-methyl-1-^{13}C]methionine (1.8 μmol/kg/h) was given during 5 h. Arterialized blood samples of 5 ml were taken at $t=-5$, -3 and -1 min and with 10 min intervals in the first hour and with 20 min intervals until 5 h. After sampling, heparin blood was immediately put on ice, the plasma was separated and frozen at −30°C.

2.2. Instrumentation

Samples were analysed by gas chromatography–mass spectrometry (GC–MS) using a HP Engine (Hewlett–Packard, Palo Alto, CA, USA) which consisted of a 5890 series II gas chromatograph and a 5989B mass spectrometer. The heated split/splitless injector (285°C) was connected through a precolumn of methyl deactivated fused-silica, 1 m×0.53 mm (I.D.) with a CP Sil 88 CB (WCOT fused-silica), 25 m×0.32 mm (I.D.)×0.20 μm (film thickness) GC column (Chrompack International, Middelburg, The Netherlands). Helium was used as carrier gas with a constant flow of 2.1 ml/min. Typically 1 μl of sample was injected with the GC programmed to increase from 80°C (1 min) to 200°C with a rate of 15°C/min and with 8°C/min to 240°C where the temperature was kept constant for 6 min.

The mass spectrometer was operated with an interface temperature of 245°C and a source temperature of 200°C using negative chemical ionization (NCI) conditions with NH_3 as the moderating gas. The acetyl-3,5-bis–trifluoromethyl benzyl derivatives of methionine, [1-^{13}C]methionine and [$^{2}H_3$-methyl-1-^{13}C]methionine were monitored at m/z −190.05, m/z −191.06 and m/z −194.08, respectively; the diacetyl-3,5-bis–trifluoromethyl benzyl derivatives of homocysteine and [1-^{13}C]homocysteine were monitored at m/z −218.05 and m/z −219.05, respectively, using dwell times of 70 ms. Calibration curves obtained by measurement of standard mixtures containing weighed amounts of tracer and tracee were used to correct for minor instrument variation.

2.3. Chemicals

DL-Dithiothreitol (99%) and L-methionine were obtained from Sigma (St. Louis, MO, USA); acetic anhydride (95%), hydrochloric acid (36–38%) and hydrobromic acid (48%) from J.T. Baker (Deventer, The Netherlands); hexane, methanol, sodium hydroxide and potassium dihydrogenphosphate were from Merck (Darmstadt, Germany); diisopropylethylamine from Janssen Chimica (Geel, Belgium); acetonitrile and ethylacetate from Vel (Leuven, Belgium); ammonia (24–26%) from Brocacef (Maarssen, The Netherlands); 3,5-bis–trifluoromethylbenzylbromide from Fluorochem (Old Glossop, UK).

The anion-exchange resin AG 1-X8 (100–200 mesh, chloride form) was purchased from BioRAD Laboratories (Richmond, CA, USA); L-[1-^{13}C]-methionine (99 AP) from Isotec (Miamisburg, OH,

USA); L-[1-^{13}C]methionine-[*S*-methyl-$^{2}H_3$] (1-^{13}C, 99 AP; 99 AP $^{2}H_1$) from MassTrace (Woburn, MA, USA).

2.4. Derivatization

For isotopic analysis methionine was analysed as the acetyl-3,5-bis–trifluoromethylbenzyl derivative and (total) homocysteine as the diacetyl-3,5-bis–trifluoromethylbenzyl derivative.

Plasma samples were purified with anion-exchange chromatography according to Stabler et al. [20]. To 500 μl of plasma, 1 ml of H_2O and 50 μl of 1 *M* NaOH containing 10 mg/ml of dithiothreitol were added. The mixture was shaken vigorously and kept at 40°C for 30 min. The sample was applied to the anion-exchange column (AG 1-X8 resin, 100–200 mesh, chloride form) and washed three times with 3 ml of H_2O and once with 2 ml of methanol. The analytes were eluted with 1 ml of 0.5 *M* HCl in methanol and dried under nitrogen at 40°C. After addition of 0.5 ml H_2O, 1 ml phosphate buffer (pH 10.5; 1 *M*) and 100 μl of 1 *M* NaOH containing 10 mg/ml of dithiothreitol, the mixture was shaken and kept at 40°C for 15 min. Thereafter, 50 μl of acetic anhydride was added and the mixture was kept at 40°C for another 15 min. The analytes were extracted with 3 ml of ethyl acetate and 250 μl of 6 *M* hydrochloric acid. The organic layer was dried under nitrogen at 40°C. To the dried sample 110 μl of 3,5-bis–trifluoromethylbenzylbromide in acetonitrile (10%) and 10 μl of diisopropylethylamine were added. After mixing, the sample was kept at 40°C for 15 min. The analytes were extracted with 1 ml of hexane and 200 μl of 0.5 *M* hydrochloric acid. The organic layer was washed with 1 ml of H_2O. The hexane layer was stored in capped vials at 5°C until GC–MS analysis.

2.5. Preparation of standards

Labelled methionine enriched standards were prepared by (i) weighing an amount of labelled L-methionine; (ii) addition of a weighed amount of unlabelled L-methionine to obtain the required enrichment. The combined methionine was dissolved and mixed completely in (heated) water. Thereafter, the mixture was dried and the resulting powder was stored at room temperature under nitrogen. The procedure was repeated to obtain independent standards with different enrichments expressed as percentages of tracer to tracee ratio (TTR) for [1-^{13}C]methionine (0.0, 1.1, 2.1 and 3.1%) and [$^{2}H_3$-methyl-1-^{13}C]methionine (0.0, 1.3, 3.1, 7.1 and 12.1%).

[1-^{13}C]homocysteine enriched homocysteine standards were obtained by chemical conversion of [1-^{13}C]-enriched methionine standards (see Fig. 1) by modification of a method of Baernstein [21] used for determining methionine in proteins. A sample of methionine standard was dissolved in hydrobromic acid (48%) with 1% H_3PO_2 and kept at 120°C overnight. After drying the mixture (95°C, 4 h) the ring of the resulting thiolactone of homocysteine hydrobromide was opened with ammonia (25%). Thereafter, homocysteine was derivatized with acetic anhydride and 3,5-bis–trifluoromethylbenzylbromide as described above. The procedure was repeated to obtain the required calibration standards (0.0, 1.5, 2.5, 3.4, 5.7 and 7.6% TTR).

2.6. Statistics

Enrichments were expressed as TTR (%) throughout this paper [22]. Data were expressed as a mean±SD. Calibration curves were analysed using linear regression (least squares).

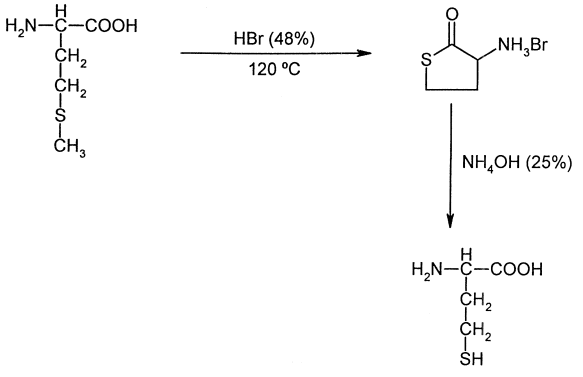

Fig. 1. Chemical conversion scheme of methionine into homocysteine.

3. Results and discussion

3.1. Mass spectra

The 3,5-bis–trifluoromethylbenzyl (BTFMB) derivatives have excellent electron capture capacities and therefore are well suited for negative chemical ionisation (NCI). NCI spectra of the (di)acetyl-3,5-bis–trifluoromethylbenzyl derivatives of homocysteine and methionine are shown in Fig. 2. In both cases the loss of the BTFMB moiety resulted in the base peak of the spectrum. The resulting fragments contain all the atom positions for the points of labelling; therefore, these fragments were chosen for selected ion monitoring.

3.2. Validation

3.2.1. Linearity

The calibration curve as shown in Fig. 3 was typical for [1-^{13}C]homocysteine, as well as for similar calibration curves for [1-^{13}C]methionine and [$^{2}H_3$-methyl-1-^{13}C]methionine (results not shown). Good linear relationships were obtained in the respective working ranges for calculated TTR versus measured TTR on 4 separate days over a period of 0.5 years: [1-^{13}C]methionine: $r=0.9993\pm0.0007$, slope$=0.99\pm0.02$ (enrichment range 0–3% TTR); [$^{2}H_3$-methyl-1-^{13}C]methionine: $r=0.9998\pm0.0002$, slope$=0.99\pm0.02$ (enrichment range 0–12% TTR) and [1-^{13}C]homocysteine: $r=0.9992\pm0.0007$, slope$=1.00\pm0.02$ (enrichment range 0–8%).

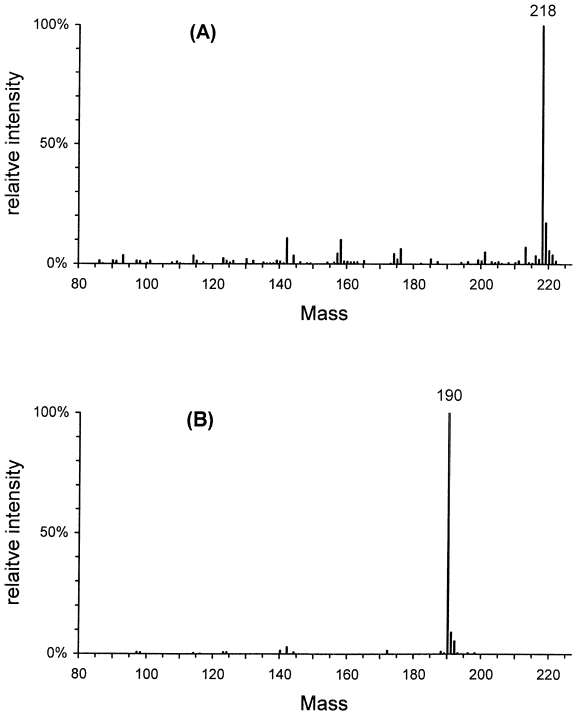

Fig. 2. NCI mass spectra of the (di)acetyl-3,5-bis–trifluoromethylbenzyl derivatives of unlabelled homocysteine (A) and methionine (B).

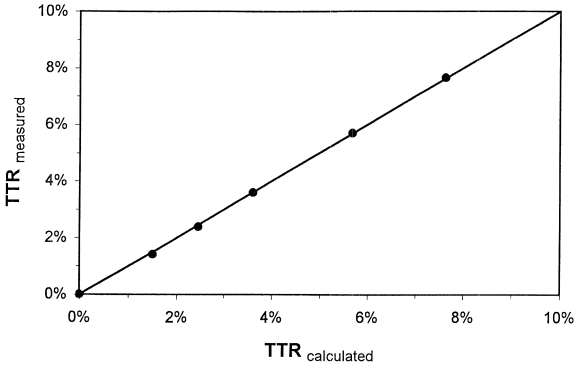

Fig. 3. Typical calibration curve for [1-^{13}C]homocysteine (slope$=$ 1.0004, $r^2=0.9997$).

3.2.2. Precision and accuracy

In comparison with the existing methods for a combined derivatization of methionine and homocysteine using e.g., *t*-butyldimethylsilyl derivatives [20] the method described results in simple isotopic clusters at the masses for single ion monitoring. The much lower natural abundances for the [m+1] ions (e.g. for natural homocysteine the [m+1]/m ratio is 0.1053 instead of 0.3834 for the *t*-butyldimethylsilyl derivative) allow for a much higher precision at low enrichments.

The precision for the determination of [1-^{13}C]methionine, [$^{2}H_3$-methyl-1-^{13}C]methionine and [1-^{13}C]homocysteine in standards and plasma was not significantly different.

The intra-day precision for the ratios of area under the curve in the ion-chromatograms were measured with C.V.s $<1\%$ ($N=3$) for [1-^{13}C]methionine and [1-^{13}C]homocysteine. Since the ratio for [$^{2}H_3$-methyl-1-^{13}C]methionine at natural abundance is

close to zero, the C.V.s ranged from 20 to 0.8% for the working range. Therefore, the respective plasma enrichments could be determined with an intra-day precision for respectively [1-^{13}C]methionine: TTR (%)±0.10% (enrichment range 0–3%); [$^{2}H_3$-methyl-1-^{13}C]methionine: TTR (%)±0.10% (enrichment range 0–12%); [1-^{13}C]homocysteine: TTR (%)±0.12% (range: 0–8%).

The inter-day precisions (over a period of 1 year, ratios of areas under the curve in ion-chromatograms) were measured with C.V.s <2%. The respective plasma enrichments could be determined with a inter-day precision for, respectively, [1-^{13}C]methionine: TTR (%)±0.15% (enrichment range 0–3%, N=18); [$^{2}H_3$-methyl-1-^{13}C]methionine: TTR (%)±0.15% (enrichment range 0–12%, N=10); [1-^{13}C]homocysteine: TTR (%)±0.16% (enrichment range: 0–8%; N=5).

As a measure of the accuracy we used the ratio between TTR determined and TTR based on weighed amounts, expressed as a percentage. The mean value of standards in the respective enrichments ranges is given for measurements on 4 separate days. The accuracies were respectively for [1-^{13}C]methionine: 100.1%±1.1% (enrichment range 1–3%, N=4); [$^{2}H_3$-methyl-1-^{13}C]methionine: 99.5%±1.6% (enrichment range 3–12%, N=4); [1-^{13}C]homocysteine: 100.5%±1.3% (enrichment range: 2–8%; N=4).

As a result, plasma enrichments of the three tracers can be determined in their working ranges better than TTR (%)±0.2%.

3.3. Stability

The derivatized standards of methionine and homocysteine stored in hexane, under nitrogen at 5°C did not show any decay over a period of 1 year. Since underivatised standards were stored with fixed enrichments, fresh standard solutions with the same enrichment could be prepared from existing stock at any time if necessary.

3.4. Degree of conversion

The degree of chemical conversion of methionine into homocysteine was approximately 2%. For the purpose of [1-^{13}C]-enrichment calibration standards improvement of the relative yield was irrelevant because amounts were redundant. Therefore, we did not try to improve the degree of conversion (for example by using hydroiodic acid). Enrichments measured for homocysteine and the corresponding methionine standards (measured at the same concentration level) were not significantly different (paired t-test, P<0.05).

3.5. Application in stable isotope studies

The method was applied in primed constant infusion studies with [$^{2}H_3$-methyl-1-^{13}C]methionine. Fig. 4 shows representative ion chromatograms for the determination of enrichments of [1-^{13}C]-methionine, [$^{2}H_3$-methyl-1-^{13}C]methionine and [1-^{13}C]homocysteine in human plasma. The signal-to-noise ratio (S/N) for the ions with the lowest intensity ([$^{2}H_3$-methyl-1-^{13}C]methionine and [1-^{13}C]homocysteine at natural abundance) was always >100. For handling convenience we used plasma samples of 500 μl. However, the signal-to-noise ratios and the size of the end volumes, containing the derivatized compounds, allow for miniaturisation of the method.

Typical enrichment curves during a primed constant infusion in a human volunteer are shown in Fig. 5.

The first results of our studies concerning homo-

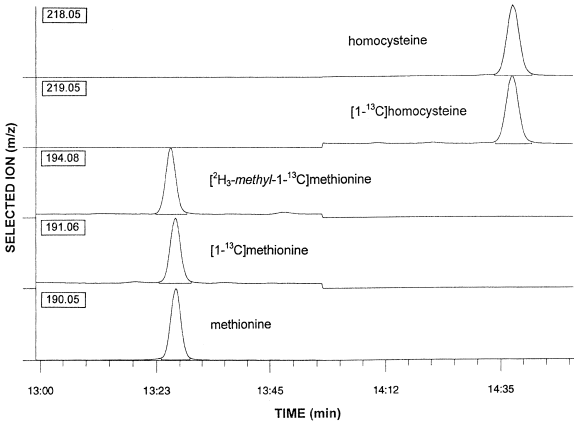

Fig. 4. Selected ion chromatograms (normalised peaks) for derivatized methionine and homocysteine in human plasma.

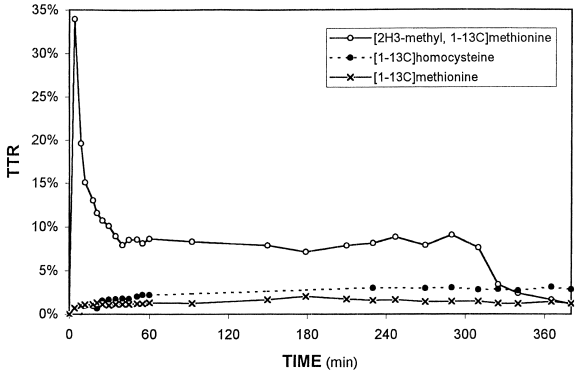

Fig. 5. Time course of the TTR of [1-^{13}C]methionine, [$^{2}H_3$-methyl-1-^{13}C]methionine and [1-^{13}C]homocysteine during a primed constant infusion of [$^{2}H_3$-methyl-1-^{13}C]methionine (sampling intervals of 5 min during the first hour, infusion stopped at t=300 min).

cysteine remethylation, methionine transmethylation and oxidation in end-stage renal disease are published elsewhere [19]. Oxidation of [$^{2}H_3$-methyl-1-^{13}C]methionine was measured on basis of $^{13}CO_2$ enrichments in expired air using isotope ratio mass spectrometry and CO_2 production measured with indirect calorimetry as described elsewhere [23].

Using the method described, it is also possible to study the kinetics of the blood borne substances in detail by typical decay curves after a bolus injection [24]. Typically a bolus of [$^{2}H_3$-methyl-1-^{13}C]-methionine of approximately 2 μmol/kg in a healthy volunteer can be followed until tracer enrichments drop below 0.2% TTR, which will take approximately 5–6 h. Dissemination of the results of these kinetic studies using bolus administration of [$^{2}H_3$-methyl-1-^{13}C]methionine is in preparation.

4. Conclusion

Reliable [1-^{13}C]homocysteine calibration standards can be prepared by chemical conversion via the thiolactone of [1-^{13}C]methionine or [$^{2}H_3$-methyl-1-^{13}C]methionine standards.

Using anion-exchange chromatography, (di)acetyl-3,5-bis–trifluoromethylbenzyl derivatives, accurate calibration curves and gas chromatography–negative chemical ionization mass spectrometry, isotopic enrichments of [1-^{13}C]homocysteine, [1-^{13}C]-methionine and [$^{2}H_3$-methyl-1-^{13}C]methionine in plasma can be determined simultaneously with TTR (%) $<\pm 0.2\%$.

The method described is well suited to study homocysteine kinetics in vivo, using [$^{2}H_3$-methyl-1-^{13}C]methionine as infusate.

Acknowledgements

The authors thank Dr. C. van Guldener MD for his help during the in vivo experiments.

References

[1] J.D. Finkelstein, Eur. J. Pediatr. 157 (Suppl. 2) (1998) S40–S44.
[2] K.J. Storch, D.A. Wagner, J.F. Burke, V.R. Young, Am. J. Physiol. 255 (Endocrinol. Metab. 18) (1988) E322–E331.
[3] K.J. Storch, D.A. Wagner, J.F. Burke, V.R. Young, Am. J. Physiol. 258 (Endocrinol. Metab. 21) (1990) E790–E798.
[4] V.R. Young, D.A. Wagner, R. Burini, K.J. Storch, Am. J. Clin. Nutr. 54 (1991) 377–385.
[5] K.J. Storch, D.A. Wagner, V.R. Young, Am. J. Clin. Nutr. 54 (1991) 386–394.
[6] Y.-M. Yu, J.F. Burke, V.R. Young, J. Trauma 35 (1993) 1–7.
[7] H. Refsum, P.M. Ueland, Current Opinion in Lipidology 9 (1998) 533–539.
[8] A. Moustapha, K. Robinson, Coronary Artery Disease 9 (1998) 725–730.
[9] T.B. Domagala, A. Undas, M. Libura, A. Szczeklik, J. Cardiovascular Risk 5 (1998) 239–247.
[10] L.J. Seman, J.R. McNamara, E.J. Schaefer, Curr. Opin. Cardiol. 14 (1999) 186–191.
[11] G.J. Hankey, J.W. Eikelboom, Lancet 354 (1999) 407–413.
[12] M.E. Lee, H. Wang, Trends Cardiovasc. Med. 9 (1999) 49–54.
[13] J.B. Ubbink, W.J.H. Vermaak, S. Bissbort, J. Chromatogr. 565 (1991) 441–446.
[14] I. Fermo, C. Arcelloni, G. Mazzola, A. Dángelo, R. Paroni, J. Chromatogr. B 719 (1998) 31–36.
[15] H. Kataoka, K. Takagi, M. Makita, J. Chromatogr. B 664 (1995) 421–425.
[16] S.-W. Myung, M. Kim, H.-K. Min, E.-A. Yoo, K.-R. Kim, J. Chromatogr. B 727 (1999) 1–8.
[17] V. Ducros, D. Schmitt, G. Pernod, H. Faure, B. Polack, A. Favier, J. Chromatogr. B 729 (1999) 333–339.
[18] J.B. Ubbink, R. Delport, R. Riezler, W.J. Hayward, Clin. Chem. 45 (1999) 670–675.
[19] C. van Guldener, W. Kulik, R. Berger, D.A. Dijkstra, C. Jakobs, D.-J. Reijngoud, A.J.M. Donker, C.D.A. Stehouwer, K. De Meer, Kidney Int. 56 (1999) 1064–1071.

[20] S.P. Stabler, J. Lindenbaum, D.G. Savage, R.H. Allen, Blood 81 (1993) 3404–3413.
[21] H.D. Baernstein, J. Biol. Chem. 106 (1934) 451–456.
[22] R.R. Wolfe, Radioactive and Stable Isotope Tracers in Biomedicine. Principle and Practice of Kinetic Analysis, Wiley-Liss, New York, 1992.
[23] D.E. Matthews, K.J. Motil, D.K. Rohrbaugh, J.F. Burke, V.R. Young, D.M. Bier, Am. J. Physiol. 238 (Endocrinol. Metab. 1) (1980) E473–E479.
[24] C. Cobelli, G. Toffolo, D.M. Bier, R. Nosadini, Am. J. Physiol. 253 (Endocrinol. Metab. 16) (1987) E551–E564.