papers and notes on methodology

Contribution of 3-hydroxyisobutyrate to the measurement of 3-hydroxybutyrate in human plasma: comparison of enzymatic and gas-liquid chromatography-mass spectrometry assays in normal and in diabetic subjects

Angelo Avogaro and Dennis M. Bier

Metabolism Division, Departments of Medicine and Pediatrics, Washington University School of Medicine, St. Louis, MO 63110

Abstract In this study we employed a capillary gas-liquid chromatographic-mass spectrometric (GLC-MS) method to measure the plasma concentrations of 3-hydroxybutyrate (3-OHB) and 3-hydroxyisobutyrate (3-OHIB) in overnight fasted diabetic subjects and in normal subjects. Plasma contents of 3-hydroxybutyrate measured in this fashion were identical to those obtained by enzymatic assay using a commercial preparation of betahydroxybutyrate dehydrogenase, indicating no significant contamination of this enzyme preparation with 3-hydroxyisobutyrate dehydrogenase. In normal individuals, plasma 3-OHIB concentration was 21 ± 2 μM in the overnight fasted state and was highter in diabetic subjects (38 ± 5 μM) and in subjects fasted for 72 h (97 ± 4 μM). In the postabsorptive state, 3-OHIB was 33% the concentration of 3-OHB in normals and 17% that of 3-OHB in the diabetics. —**Avogaro, A., and D. M. Bier.** Contribution of 3-hydroxyisobutyrate to the measurement of 3-hydroxybutyrate in human plasma: comparison of enzymatic and gas-liquid chromatography-mass spectrometry assays in normal and in diabetic subjects. *J. Lipid Res.* 1989. **30:** 1811-1817.

Supplementary key words ketone bodies • ketones • 3-hydroxybutyrate dehydrogenase • valine • GLC-MS • stable isotopes • carbon-13

The ketone bodies, acetoacetate (AcAc) and 3-hydroxybutyrate (3-OHB), can dramatically increase in blood in response to different physiological and pathological circumstances such as fasting (1) and poorly controlled diabetes mellitus (2). Consequently, a number of methods for the determination of these important metabolic fuels have been developed since the availability of semi-pure 3-hydroxybutyrate dehydrogenase (EC1.1.1.30) (3-7). Recently, however, Worrall et al. (8) have shown that commercially available 3-hydroxybutyrate dehydrogenase prepared from *Rhodopseudomonas spheroides* contains the contaminant, 3-hydroxyisobutyrate dehydrogenase.

An intermediate of valine metabolism, 3-hydroxyisobutyrate (3-OHIB), has been shown to circulate in plasma (9), has been quantified in the dog (10) and rat (11), and has been found in the urine of normal individuals and diabetic subjects during ketoacidosis (11). Therefore, it is possible that reported values for plasma 3-OHB, usually determined enzymatically, have been overestimated to a degree equal to the plasma content of 3-OHIB. Using a new GLC-MS method that separates plasma 3-OHB from 3-OHIB, Des Rosiers, et al. (10) demonstrated that this is indeed the case in the dog. To our knowledge, there is no information on the extent of this overestimation in man.

In this report, we describe an additional method for simultaneously measuring the specific plasma concentrations of AcAc, 3-OHB, and 3-OHIB and assessing the degree of inaccuracy caused by 3-OHIB when measuring 3-OHB using enzymatic methods.

MATERIALS AND METHODS

Materials

GLC pure diethylether and ethylacetate, perchloric acid, and potassium hydroxide were purchased from Sigma Chemical Company Co. (St. Louis, MO). N-Methyl-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA) was purchased from Regis (Morton Grove, IL). The GLC

Abbreviations: AcAc, acetoacetate; 3-OHB, 3-hydroxybutyrate; 3-OHIB, 3-hydroxyisobutyrate; MTBSTFA, N-methyl-t-butyldimethylsilyltrifluoroacetamide; GLC-MS, gas-liquid chromatography-mass spectrometry.

stationary phase, OV-1, was obtained from Supelco (Bellefonte, PA) and a DB-17 capillary column was obtained from J and W (Folsom, CA). Acetoacetate lithium salt, DL-3-hydroxybutyrate, methyl-(+)-3-hydroxy-3-methylpropionate (3-OHIB-Me), 3-hydroxybutyrate dehydrogenase (Grade V) prepared from *Rhodopseudomonas spheroides*, and the other reagents required for the enzymatic assay of acetoacetate and 3-hydroxybutyrate were purchased from Sigma (St. Louis, MO). The 3-OHIB used as a GLC standard for quantitation of plasma 3-OHIB was prepared by hydrolysis of the 3-OHIB methyl ester.

D-(−)hydroxy[1,2,3,4-$^{13}C_4$,2,2,3-$^{2}H_3$]butyrate (M_7-3OHB) and D-(−)hydroxy[1,2,3,4-$^{13}C_4$]butyrate ($^{13}C_4$-3OHB) were purchased from Tracer Technologies, Inc. (Somerville, MA). Chemical and isotopic purities of the labeled ketones were confirmed by conventional mass spectrometric and enzymatic analysis.

Subjects

After written informed consent was obtained according to approved procedures of the Washington University Human Studies Committee, plasma samples were obtained after an overnight fast from 8 normal volunteers (aged 30 ± 1 y, mean ± SE), and from 14 diabetic subjects (32 ± 3 y) on their usual insulin regimens, and from 4 normal individuals (26 ± 1 y) fasted for 3 days as part of another research protocol. The postabsorptive plasma glucose (mean ± SE) of the normal volunteers was 4.72 ± 0.06 mM, of the 3-day fasted subjects was 3.28 ± 0.06 mM, and of the diabetics was 9.00 ± 0.95 mM. In the latter group glycosylated hemoglobin (HbA_1) averaged 9.3 ± 0.4% at the time the samples were obtained (normal range 4–7%).

All samples were collected in dry heparinized glass tubes and kept on ice. The blood was immediately centrifuged at 4°C. The aliquots for the determination of acetoacetate were assayed immediately. The aliquots for the determination of 3-OHB and 3-OHIB were stored at −80°C until assay, generally within 5–7 days. In our hands, plasma samples stored at −80°C for periods of 10 days and 30 days show decrements in hydroxybutyrate content of less than 5% and 8%, respectively.

Enzymic fluorometric continuous-flow assay

An aliquot of 1.5 ml of plasma was placed in a preweighed tube and thoroughly mixed with 5 ml 5% (wt/vol) perchloric acid. The supernate was removed and used for analysis of 3-OHB according to the method of Lloyd et al. (5) and for measurement of AcAc by the method of Price, Lloyd, and Alberti (4).

Spectrophotometric and fluorometric enzymic methods

An aliquot of 0.6 ml of plasma was deproteinized with 0.6 ml perchloric acid and neutralized with 5% KOH. The ketone bodies were assayed spectrophotometrically according to the method of Williamson and Mellanby (3) and fluorometrically by the method of Persson (7).

Gas-liquid chromatographic-mass spectrometric analysis

To each 0.5-ml aliquot of deproteinized supernatant, 19.4 nmol of M_7-3OHB was added as internal standard. Each aliquot was divided into three portions: the first was used subsequently in an experiment to simulate an in vivo tracer study (see Calculations and Results), and the second and third were processed as follows. The second aliquot was poured into a 4-ml cuvette containing phosphate buffer (0.1 M, pH 6.8) and suitable amounts of NADH and 3-hydroxybutyrate dehydrogenase were then added. The decrease in extinction at 340 nm was followed by UV spectrophotometry. This procedure converted the sample AcAc to 3-OHB. Once the reduction reaction reached completion, both this reduced sample and the third nonreduced sample were each transferred to separate 7-ml screwcapped tubes. Two drops of 3 N HCl were added to each tube to regenerate the ketoacids which were then extracted once into 3 ml of ethylacetate. The extract was dried and the ketones were converted to the tertbutyldimethylsilyl derivatives with MTBSTFA according to the procedure of Miles et al. (12). Additional sets of plasma were spiked with 3-OHB (final concentrations: 30, 59, 118, 237, 475, and 950 μM) and AcAc (final concentrations: 34, 68, 137, 275, 550, and 1100 μM) to determine the assay precision and recovery for the two ketones.

Subsequently, the tert-butyldimethylsilyl ketones were quantified by selected ion monitoring quadrupole GLC-MS on two systems. First, after isothermal separation at 150°C on a 2 m × 2 mm column packed with 3% OV1 according to the method of Miles et al. (12), and secondly after 105°C isothermal separation on a 25-m DB 17 fused silica capillary column using helium as a carrier gas. On the former packed column, 3-OHB and 3-OHIB co-elute whereas they are resolved completely by the latter capillary GLC separation. Following electron impact ionization at 70 eV, ions at *m/z* 275 and 282 were monitored to quantify naturally occurring 3-OHB, 3-OHIB, and the internal standard (M_7-3OHB), respectively. Prior to each analytical run, standard mixtures of 3-OHB and 3-OHIB spiked with known quantities of M_7-3OHB were run to construct a standard curve. The linearity of this curve was maintained over a wide range of natural 3-OHB concentrations both when derivatized directly and when the standard solutions were subjected to the complete extraction procedure.

The intra-assay measurement precision (SD/mean × 100) of the ion current ratio *m/z* 282/275 in the standard solutions was ± 1% of the observed value. The inter-assay measurement precision averaged 3.3% of the measured ratio.

Calculations

In the plasma samples, concentrations of 3-OHB, and 3-OHIB were calculated from the respective ion current ratios in the manner of Bougnères et al. (13) and expressed according to the convention of Cobelli et al. (14).

Using the standard solutions described above containing a known quantity of M_7-3OHB and varying amounts of natural 3-OHB, a standard curve was defined by the equation:

$$Rc = Ro + k(n0/n7) \qquad Eq.\ 1)$$

where Rc was the observed 275/282 ion current ratio of the calibration standard; Ro was the ion current ratio of the M_7-3OHB internal standard alone (virtually zero); k was the slope of the line; and n0 and n7 were the absolute amounts of natural and labeled internal standard in the calibration mixture. Since Rc and Ro were measured and n0 and n7 were known, equation 1 can be solved for k. The amount of 3-OHB in the plasma sample (n0) was then calculated from the ratio of the plasma aliquot not subject to enzymatic reduction according to the following:

$$ns_1 = (n7/n0)\ (Rs - Ro). \qquad Eq.\ 2)$$

The total amount of unlabeled 3-OHB (including the reduced AcAc) was then calculated in the corresponding "reduced" aliquot subjected to enzymatic conversion of AcAc to 3-OHB according to the equation:

$$ns_2 = (n7/n0)\ (Rs - Ro). \qquad Eq.\ 3)$$

The amount of AcAc is the given by the difference:

$$ns_2 - ns_1. \qquad Eq.\ 4)$$

The results were corrected for sample volume and dilution factors and expressed as μmol/l.

Calculation of plasma 3-OHIB was performed in an identical fashion using a standard curve constructed for 3-OHIB and the same ion current ratios, but taking advantage of the complete separation of 3-OHIB from 3-OHB on the DB-17 capillary GLC column. Values for the 3-OHIB were further corrected by a factor of 1.24 for the coefficient of extraction of plasma 3-OHIB relative to that of 3-OHB (1.24 ± 0.01 determined on five different samples).

In order to simulate an in vivo experiment in which $^{13}C_4$-3OHB was used as a tracer, varying known amounts of $^{13}C_4$-3OHB were added to the first aliquot (see above) which also contained M_7-3OHB internal standard. Using the principles above and the ion current ratio *m/z* 279/275, the plasma $^{13}C_4$-3OHB isotopic enrichment was calculated (13, 14) in an analogous fashion.

RESULTS

Table 1 shows the measured plasma 3-OHB and AcAc levels for the four different methods tested. In the unspiked samples, enzymatic continuous flow fluorometry overestimated the content of 3-OHB and underestimated the AcAc concentration compared with the other methods which gave identical values. Overall recovery in the spiked samples was essentially 100% with all assays. However, at plasma 3-OHB concentrations of 118 μM or less, the packed column GLC-MS method overestimated plasma 3-OHB concentration by 117–136%, presumably due to the simultaneous elution of 3-OHIB which is estimated as approximately 20 μM from the difference in measured values using the capillary and the packed column GLC assays. At higher 3-OHB concentrations, assay variance obscures this difference.

Overall assay precisions (SD/mean × 100) for determination of 3-OHB and AcAc, respectively, were 5.5 ± 3.9% and 6.6 ± 2.4% (continuous flow fluorometry), 10.9 ± 4.4 and 8.6 ± 5.5% (spectrophotometry), 8.7 ± 4.7% and 9.6 ± 4.3% (fluorometry), and 1.6 ± 0.3% and 8.6 ± 4.4% (capillary column GLC-MS). The precision of measurement of plasma 3-OHB by capillary column GLC-MS was significantly ($P < 0.05$) better than that of the other methods. The assay precision of capillary GLC-MS determination of AcAc was similar to that of the other methods, possibly because calculation of Ac-Ac is based on differences between two measured values. The precision of 3-OHB measurement by packed column GLC-MS was 2.27 ± 1.74%, near the range of the precision achieved with capillary GLC-MS.

There was excellent correlation between fluorometric (Y) and capillary GLC-MS (X) measurement of 3-OHB ($Y = 15.37 \pm 1.18X$, $r^2 = 0.993$). However, the slope of 1.18 suggested potential contribution of 3-OHB to the fluorometrically measured value due to contamination of 3-hydroxybutyrate dehydrogenase with 3-hydroxyisobutyrate dehydrogenase. Therefore, two sets of standard solutions were prepared containing equal quantities of either 3-OHB or of 3-OHIB. Both were then measured fluorometrically using the Sigma 3-hydroxybutyrate dehydrogenase preparation. The results are shown in **Table 2**. Under the assay conditions used in this study, virtually no 3-OHIB was measured by the commercially prepared semi-purified enzyme.

Plasma samples obtained from the eight postabsorptive normal subjects were then analyzed for 3-OHB concentration by packed column GLC-MS, by capillary column GLC-MS, and by fluorometric enzymatic assay (**Table 3**). The respective values (mean ± SE) were 84 ± 20 μM, 63 ± 20 μM, and 64 ± 19 μM. Plasma 3-OHIB measured

TABLE 1. Assay precision and recovery

Analytical Method	Basal [3-OHB]	Concentration of 3-OHB Added to the Basal Sample (μM) 30	59	118	237	475	950	Recovery
		3-OHB concentration observed						
	μM	μM (± SD)						%
Continuous flow fluorometry	60	93 (10)	114 (11)	169 (6)	303 (9)	502 (10)	888 (20)	96 (6)
Spectrophotometry	34	74 (12)	102 (15)	144 (18)	247 (25)	458 (31)	866 (45)	98 (11)
Fluorometry	34	58 (9)	83 (10)	164 (17)	276 (16)	499 (30)	945 (25)	97 (7)
Packed column GLC-MS	34	87 (5)	115 (2)	178 (2)	292 (6)	504 (9)	935 (11)	113 (15)
Capillary GLC-MS	34	66 (1)	94 (1)	150 (1)	280 (6)	512 (8)	943 (13)	100 (3)
		Concentration of AcAc Added to the Basal Sample (μM) 34	68	137	275	550	1100	
	Basal [AcAc]	AcAc concentration observed						
Continuous flow fluorometry	16	48 (5)	82 (6)	161 (12)	335 (21)	600 (24)	1194 (51)	104 (7)
Spectrophotometry	26	51 (9)	82 (10)	150 (13)	310 (17)	530 (24)	1092 (36)	93 (7)
Fluorometry	26	54 (8)	87 (12)	166 (18)	319 (25)	588 (39)	1160 (45)	99 (6)
Capillary GLC-MS	26	52 (8)	83 (9)	143 (14)	304 (25)	541 (27)	1103 (33)	93 (6)

in the same plasma samples by capillary GLC-MS averaged 21 ± 2 μM, precisely the overestimation of 3-OHB concentration determined by the packed column GLC-MS assay in which both ketones co-elute.

In **Fig. 1**, the 3-OHIB concentrations measured in postabsorptive normal adults are shown along with the plasma 3-OHIB contents of the 14 postabsorptive diabetic subjects, and those of the 4 normal individuals fasted for 72 h. In normal postabsorptive adults, plasma 3-OHIB averaged 21 ± 2 μM, one-third the concentration of 3-OHB (63 ± 20 μM). Plasma 3-OHIB concentrations were significantly higher in the post-absorptive diabetic (38 ± 5 μM) and in the 3-day fasted individuals (97 ± 4 μM) but represented significantly smaller fractions of total 3-hydroxybutyrates since 3-OHB averaged 226 ± 106 μM in the diabetic and 2507 ± 235 μM in the fasted adults.

Table 3 also reports the apparent and the specific enrichments of $^{13}C_4$-3OHB obtained with packed column GLC-MS and with capillary GLC-MS assays, respectively. As expected from the co-elution of 3-OHIB and 3-OHB on the packed GLC column, the apparent $^{13}C_4$-3OHB isotopic enrichment was underestimated using this method.

Fig. 2 shows the difference between the true plasma isotopic enrichment of $^{13}C_4$-3OHB measured by capillary GLC-MS and the spuriously low plasma $^{13}C_4$-3OHB enrichment determined by packed column GLC-MS in the aliquots of plasma to which a known amount of $^{13}C_4$-3OHB was added in order to simulate the results of an in vivo tracer experiment. When plasma 3-OHB concentrations were

TABLE 2. Assessment of contribution of 3-hydroxyisobutyrate to measurement of 3-hydroxybutyrate using commercially prepared 3-hydroxybutyrate dehydrogenase

3-OHB Concentration in Standards		3-OHIB Concentration in Standards	
Expected	Observed	Expected	Observed
μM		μM	
40	42 ± 2	40	2 ± 2
80	77 ± 3	80	2 ± 2
160	156 ± 7	160	3 ± 1
320	308 ± 12	320	5 ± 2
640	623 ± 25	640	5 ± 2
1280	1214 ± 48	1280	5 ± 4

TABLE 3. Plasma 3-OHB and 3-OHIB content and $^{13}C_4$ enrichment of plasma 3-OHB in the study subjects

	Normal Subjects	Diabetic Subjects	3-Day Fasted Subjects
3-OHB + 3-OHIB (μM) (packed GLC-MS)	84 ± 20	259 ± 107[a]	3021 ± 181[a,b]
3-OHB (μM) (enzymatic assay)	64 ± 19	204 ± 88	
3-OHB (μM) (capillary GLC-MS)	63 ± 20	226 ± 106[a]	2507 ± 235[a,b]
3-OHIB (μM) (capillary GLC-MS)	21 ± 2	38 ± 5[a]	97 ± 4[a,b]
Apparent $^{13}C_4$-3OHB enrichment (packed column GLC-MS, atom % excess)	26.98 ± 3.21	16.76 ± 2.86[a]	1.36 ± 0.39[a,b]
Specific $^{13}C_4$-3OHB enrichment (capillary GLC-MS, atom % excess)	40.85 ± 4.47[c]	22.58 ± 4.64[a,c]	1.54 ± 0.72[a,b,c]

Values are given as mean ± SE.
[a]Significantly different ($P < 0.01$) from normal value.
[b]Significantly different ($P < 0.01$) from diabetic value.
[c]Significantly different ($P < 0.01$) from packed column value.

less than approximately 400 μM, the packed column GLC-MS method significantly underestimated the plasma enrichment of $^{13}C_4$-3OHB due to co-elution of 3-OHIB. In the diabetic and fasted subjects with plasma 3-OHB concentrations above the level, the relatively low 3-OHIB contamination produced little change in $^{13}C_4$-3OHB atom % excess, a result virtually identical to that found by Des Rosiers et al. in dogs (10).

DISCUSSION

In 1987 Worrall et al. (8) demonstrated that a commercially available preparation of 3-hydroxybutyrate dehydrogenase commonly used for enzymatic measurement of 3-OHB was contaminated with 3-hydroxyisobutyrate dehydrogenase. Shortly thereafter, Des Rosier et al. (10), using a capillary GLC-MS method capable of measuring both 3-OHB and 3-OHIB, showed that plasma 3-OHIB was present in substantial amounts compared with circulating 3-OHB in the dog. As a consequence, these authors (10) further demonstrated that plasma isotopic enrichment values of circulating 3-OHB during a tracer injection of labeled 3-OHB were in error unless one used a procedure to separate plasma 3-OHIB from 3-OHB. Since the dog is a species with low circulating 3-OHB levels compared to those found in man, and since Worrall et al. (8) reported values obtained from the rat hemidiaphragm preparations, we investigated the significance of these observations in man where accurate determination of plasma 3-OHB levels is important for studying the pathophysiology of ketogenesis.

Compared with a specific capillary GLC-MS assay for 3-OHB, we measured no significant difference in the concentrations of 3-OHB in human plasma or in standard solutions using a commercially available preparation of 3-hydroxybutyrate dehydrogenase and several conventional enzymatic assays. As an additional control, standard mixtures were prepared containing large quantities of 3-OHIB. Furthermore, the capillary GLC-MS assay demonstrated that significant quantities of 3-OHIB were in fact present in the plasma samples. Thus, these results suggest that 3-hydroxy-isobutyrate dehydrogenase contamination of the enzyme preparation has been eliminated by the manufacturer or that its presence is not a significant problem for measurement of human plasma 3-OHB content under assay conditions commonly employed for this purpose.

Using the capillary GLC-MS assay described above, we have also shown that circulating 3-OHIB concentrations in postabsorptive man average one-third those of 3-OHB.

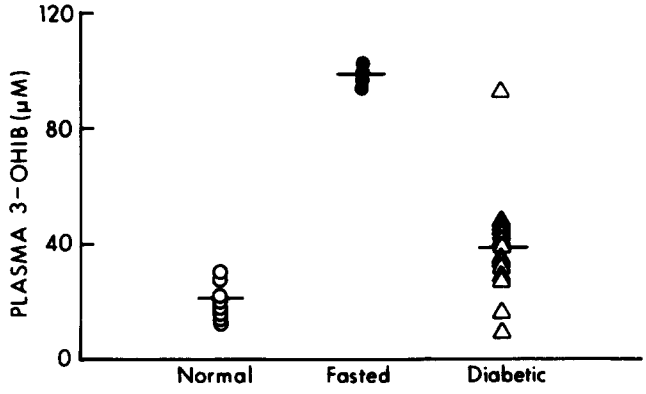

Fig. 1. Individual plasma concentrations of 3-hydroxyisobutyrate in postabsorptive normal adults (open circles), diabetic subjects (open triangles), and in adults fasted for 3 days (closed circles).

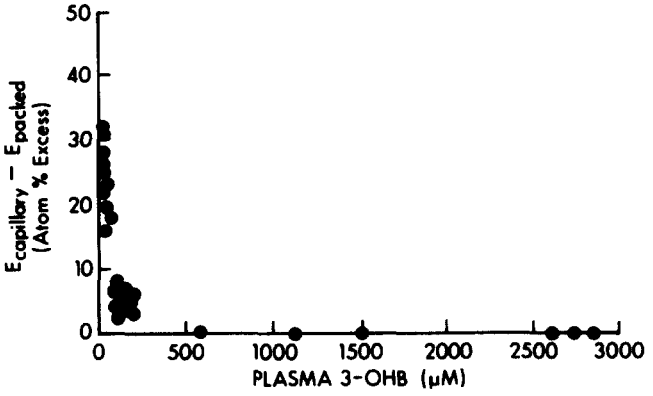

Fig. 2. The plasma enrichment of $^{13}C_4$-3OHB measured specifically by capillary GLC-MS less that determined by packed column GLC-MS (where 3-OHIB co-elutes) as a function of plasma 3-OHB concentration.

These observations confirm and extend those reported by Des Rosiers et al. (10) in the dog, a species with low circulating 3-OHB.

We have further shown that circulating 3-OHIB concentrations rise on prolonged fasting and are elevated in postabsorptive diabetic subjects. The latter observation extends Landaas' finding (11) of increased urinary excretion of 3-OHIB in diabetic individuals. Since 3-OHIB is a metabolite of valine catabolism, we speculate that its elevated plasma concentration in 3-day fasted subjects reflects augmented valine degradation secondary to net body protein breakdown. It is also conceivable that the elevated plasma 3-OHIB has a physiologic function in fasting since, at least in vitro, 3-OHIB can serve as a gluconeogenic substrate (9). We might speculate further that the increased postabsorptive plasma levels of 3-OHIB found in the diabetic subjects are likewise the result of increased protein breakdown and branched-chain amino acid catabolism which are a consequence of insulin deficiency (15). Since valine oxidation is also regulated by factors independent of body protein breakdown, our speculative hypothesis clearly requires further specific experimental testing.

As described previously by Des Rosiers et al. (10) in dogs, we have also found that estimation of $^{13}C_4$-3OHB isotopic enrichment was significantly underestimated by packed column GLC-MS in the plasma of all subjects but proportionately less so in the diabetic and the 3-day fasted samples because of the smaller relative contribution of 3-OHIB. This is depicted graphically in Fig. 2 which emphasizes the importance of using a specific capillary GLC-MS assay for quantifying stable isotopic tracer enrichment in plasma when 3-OHB concentrations are less than approximately 500 μM.

In summary then, we have shown that plasma 3-OHB can be measured reliably using a commercially available preparation of 3-hydroxybutyrate dehydrogenase. Under enzymatic assay conditions commonly used for this purpose, we have been unable to demonstrate an overestimation of 3-OHB content despite the presence of plasma 3-OHIB in the range of 14–93 μM determined by specific capillary GLC-MS assay. To our knowledge, these are the first reported values for circulating 3-OHIB in man. Finally, we have also shown that plasma 3-OHIB is elevated in fasted individuals and in subjects with type I diabetes mellitus.

We wish to thank Henri Brunengraber and Christine Des Rosiers for pointing out to us the presence of circulating 3-hydroxyisobutyrate. This work was supported in part by grants RR-00954 and AM-20579 from the National Institutes of Health. Dr. Avogaro was on leave from the University of Padova, Italy, and the recepient of a Fogarty International Research Fellowship (TW-03876) and of a NATO Fellowship (09136).

Manuscript received 26 April 1989 and in revised form 3 July 1989.

REFERENCES

1. Robinson, A. M., and D. H. Williamson. 1980. Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiol. Rev.* **60:** 143–187.
2. Fenselau, A. 1981. Ketone body metabolism in normal and diabetic man. *In* Handbook of Diabetes Mellitus: Intermediary Metabolsim and its Regulation. Vol. 3. M. Brownlee, editor. Garland, New York, NY. 143–207.
3. Williamson, D. H., and J. Mellanby. 1974. D(−)3-hydroxybutyrate. *In* Methods of Enzymatic Analysis. Vol. 4. H. U. Bergmeyer, editor. Academic Press, New York, NY. 1836–1837.
4. Price, C. B., B. Lloyd, and K. G. M. M. Alberti. 1977. A kinetic spectrophotometric assay for rapid determination of acetoacetate in blood. *Clin. Chem.* **23:** 1893–1897.
5. Lloyd, B., J. Burrin, P. Smythe, and K. G. M. M. Alberti. 1978. Enzymic fluorometric continuous-flow assays for blood glucose, lactate, pyruvate, alanine, glycerol, and 3-hydroxybutyrate. *Clin. Chem.* **34:** 1727–1729.
6. Wildenhoff, K. E. 1970. A micromethod for the enzymatic determination of acetoacetate and 3-hydroxybutyrate in blood and in urine. *Scand. J. Clin. Lab. Invest.* **25:** 171–177.
7. Persson, B. 1969. Determination of plasma acetoacetate and D-beta-hydroxybutyrate in newborn infants by an enzymatic fluorometric micromethod. *Scand. J. Lab. Invest.* **25:** 9–18.
8. Worrall, E. B., S. Gassain, D. J. Cox, M. C. Sugden, and T. N. Palmer. 1987. 3-Hydroxyisobutyrate dehydrogenase, an impurity in commercial 3-hydroxybutyrate dehydrogenase. *Biochem. J.* **241:** 297–300.
9. Letto, J., M. E. Brosnan, and J. T. Brosnan. 1986. Valine metabolism: gluconeogenesis from 3-hydroxyisobutyrate. *Biochem. J.* **340:** 902–912.
10. Des Rosiers, C., J. A. Montgomery, S. Desrochers, M. Garneau, F. David, O. A. Mamer, and H. Brunenegraber. 1988. Interference of 3-hydroxyisobutyrate with measurements of ketone body concentration and isotopic enrichment by gas chromotography–mass spectrometry. *Anal. Biochem.* **173:** 96–105.
11. Landaas, S. 1975. Accumulation of 3-hydroxyisobutyric acid, 2-methyl-3-hydroxybutyric acid and 3-hydroxyisobaleric acid in ketoacidosis. *Clin. Chim. Acta.* **64:** 143–154.
12. Miles, J., K. Schwenk, K. McClean, and M. Haymond. 1984. Determination of ketone body turnover in vivo with stable iso-

topes utilizing gas chromatography–mass spectrometry. *Anal. Biochem.* **140:** 620–626.
13. Bougnères, P. F., E. O. Balasse, P. Ferré, and D. M. Bier. 1986. Determination of ketone body kinetics using a D-(−) 3-hydroxy[4,4,4-$^2H_3$]butyrate tracer. *J. Lipid Res.* **27:** 215–220.
14. Cobelli, C., G. Toffolo, D. M. Bier, and R. Nosadini. 1987. Models to interpret kinetic data in stable isotope tracer studies. *Am. J. Physiol.* **253:** E551–E564.
15. Robert, J. J., B. Beaufrere, J. Koziet, J. F. Dexjeux, D. M. Bier, V. R. Young, and H. Lestradet. 1985. Whole body de novo amino acid synthesis in type I (insulin-dependent) diabetes studied with stable isotope-labeled leucine, alanine, and glycine. *Diabetes.* **34:** 67–73.