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QUANTITATION OF DEUTERATED AND NON-DEUTERATED PHENYLALANINE AND TYROSINE IN HUMAN PLASMA USING THE SELECTIVE ION MONITORING METHOD WITH COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

APPLICATION TO THE *IN VIVO* MEASUREMENT OF PHENYLALANINE-4-MONOOXYGENASE ACTIVITY

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SUMMARY

A specific method is described for the quantitative analysis of deuterated and non-deuterated phenylalanine and tyrosine in human plasma by gas chromatography–mass spectrometry using selective ion monitoring. From the several derivatives investigated, the N- or N,O-trifluoroacetyl methyl esters were found to be the most suitable for our purposes. DL-Phenylalanine-4-d_1 and L-tyrosine-d_7 were used as internal standards. The sensitivity of this method permits the measurement of amounts as small as *ca.* 2.5 ng/ml in plasma for both phenylalanine and tyrosine. The coefficients of variation were found to be *ca.* 1.6% ($n = 12$) for phenylalanine and 3.0% ($n = 12$) for tyrosine.

Using this method, an *in vivo* determination of phenylalanine-4-monooxygenase activity in humans is possible by loading the subjects with deuterated L-phenylalanine-d_5 (accepted as substrate by phenylalanine-4-monooxygenase E.C. 1.14.16.1) and the subsequent measuring of deuterated L-tyrosine-d_4 formed and residual L-phenylalanine-d_5.

INTRODUCTION

The tetrahydrobiopterine-dependent liver enzyme phenylalanine-4-monooxygenase (E.C. 1.14.16.1) is responsible for the conversion of phenylalanine into tyrosine in metabolic pathways. In phenylketonuria, one of the most common inborn errors of human metabolism, this enzyme shows no, or only partial, activity. In order to evaluate the remaining enzyme activity or to determine an increase in the enzyme activity after therapeutic treatment, we were interested in developing an indirect *in vivo* measurement of phenylalanine-4-monooxygenase. The indirect determination of the phenylalanine-4-monooxygenase activity in humans has already been investigated[1,2], but the specificity and reliability of these determinations are disputable. In a preliminary communication[3], we reported on an indirect *in vivo* measurement of phenylalanine-4-monooxygenase activity after loading tests with deuterated

L-phenylalanine and subsequent analysis of deuterated tyrosine in plasma with the selective ion monitoring (SIM) method.

Many methods have been described for the determination of amino acids by gas chromatography[4–25] and by combined gas chromatography–mass spectrometry (GC–MS)[28–30], using a wide selection of derivatives. For the present purpose we developed a specific method for quantitation of deuterated and non-deuterated phenylalanine and tyrosine in human plasma by the SIM technique. To guarantee high specificity and sensitivity of this method, the chosen derivatives should show a characteristic and intensive signal typical for phenylalanine or tyrosine, respectively. For the derivatization procedures acidic conditions should be avoided in order to prevent deuterium exchange.

Several different derivatives of aromatic amino acids (specially of phenylalanine and tyrosine) were studied in order to find the most suitable for our purpose. Finally, N-,(O)-trifluoroacetyl methyl esters were chosen.

EXPERIMENTAL

Materials

All chemicals were of the highest purity available and solvents were re-distilled before use.

Reference compounds were obtained from Fluka (Buchs, Switzerland).

L-Phenylalanine-d_5 (as loading material) with all deuterium atoms in the benzene ring was synthesized by Prof. P. Hemmerich, University of Konstanz, G.F.R.

The internal standard DL-phenylalanine-4-d_1 was synthesized by catalytic hydrogenation of 4-monochloro-DL-phenylalanine with palladium oxide on active carbon[26], while L-tyrosine-d_7 was obtained from Merck Sharp & Dohme, Munich (G.F.R.).

Deuterium oxide, p.a. grade (99.7 atom-% D) was obtained from Fluka and hydrochloric acid-d_1 (about 20% deuterium chloride in D_2O) from Merck (Darmstadt, G.F.R.); Dowex 50W-X8 (H^+), 200–400 mesh, was regenerated several times with sodium hydroxide solution and hydrochloric acid solution and washed before use with 1 *N* hydrochloric acid and water until neutral.

Methylation was carried out with diazomethane according to Vogel[27].

Methods

Preparation of samples. A 200-μl volume of a 5 mg per ml L-tyrosine-d_7 solution and 100 μl of a 5 mg per 100 ml DL-phenylalanine-d_1 solution as internal standards were mixed with 0.5 ml of human plasma. A 0.5-ml volume of a 4 g per 100 ml sulphosalicylic acid solution was added for deproteinization and, after repeated centrifugation (twice for 20 min at 2000 *g*), the supernatant was applied on to 2 ml of Dowex 50W-X8 (H^+) in a 20 × 0.7 cm column. After rinsing with 100 ml of water, the amino acids were eluted with 1 *N* ammonia solution. Fractions of 1 ml of column effluent were collected and measured spectrophotometrically at 240 nm, against water. The UV-positive fractions were combined and evaporated to dryness under vacuum. The remaining water was eliminated azeotropically by repeated evaporation with dichloromethane.

Derivatization. The samples prepared in this manner were derivatized to their N-,(O-)-trifluoroacetyl methyl esters by the addition of 200 μl of acetonitrile and 200 μl of trifluoroacetic anhydride (TFAA). After 10 min at room temperature and evaporation under a stream of nitrogen, diazomethane in diethyl ether was added until methylation was completed. The samples were dried again and the residues dissolved in 200 μl of acetonitrile and 40 μl of TFAA. These samples were then ready for injection.

GC–MS/SIM method. The following apparatus and conditions were used: a Carlo Erba GI 450 gas chromatograph, Fractovap 2101 AC; glass column, length 80 cm, I.D. 2 mm, with 2.5% SE-30 on Chromosorb G, 80–100 mesh; injection block, 250°; carrier gas, helium, pre-pressure 1.0 kp/cm^2; oven temperature, 150° for phenylalanine and 165° for tyrosine; Vacuum Generators mass spectrometer, Micromass 16 F with Data System 2000; separator, single jet, 260°; ion source, 220°; 20 eV; 50 μA; SEV, 25 kV.

RESULTS AND DISCUSSION

Figs. 1 and 3a show the mass spectra of the various derivatives of phenylalanine investigated, while Figs. 2 and 4a show corresponding results for tyrosine. The

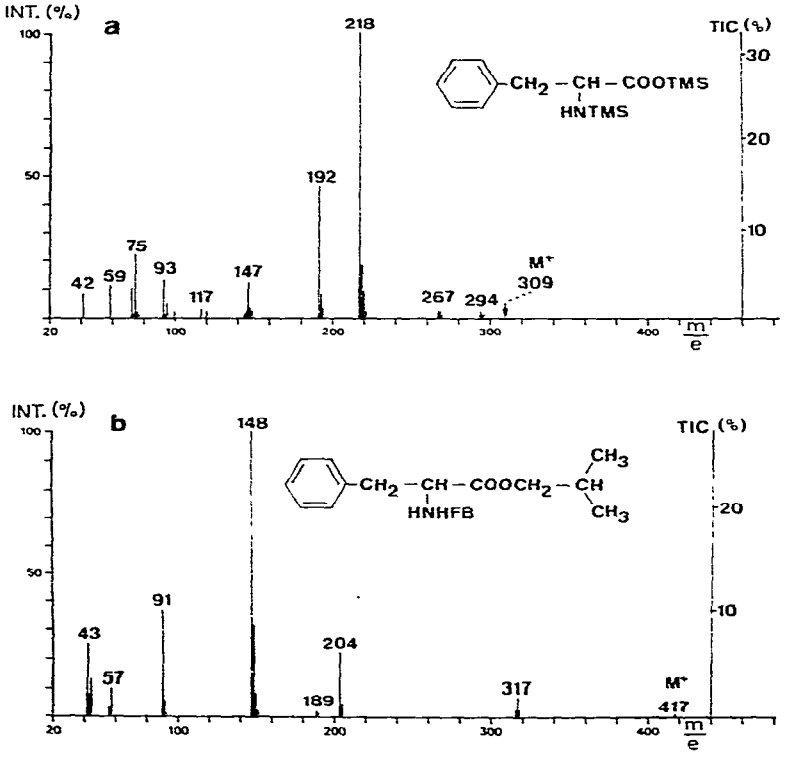


Fig. 1. Mass spectra of phenylalanine: (a) N-trimethylsilyl trimethylsilyl ester; (b) N-heptafluorobutyryl isobutyl ester.

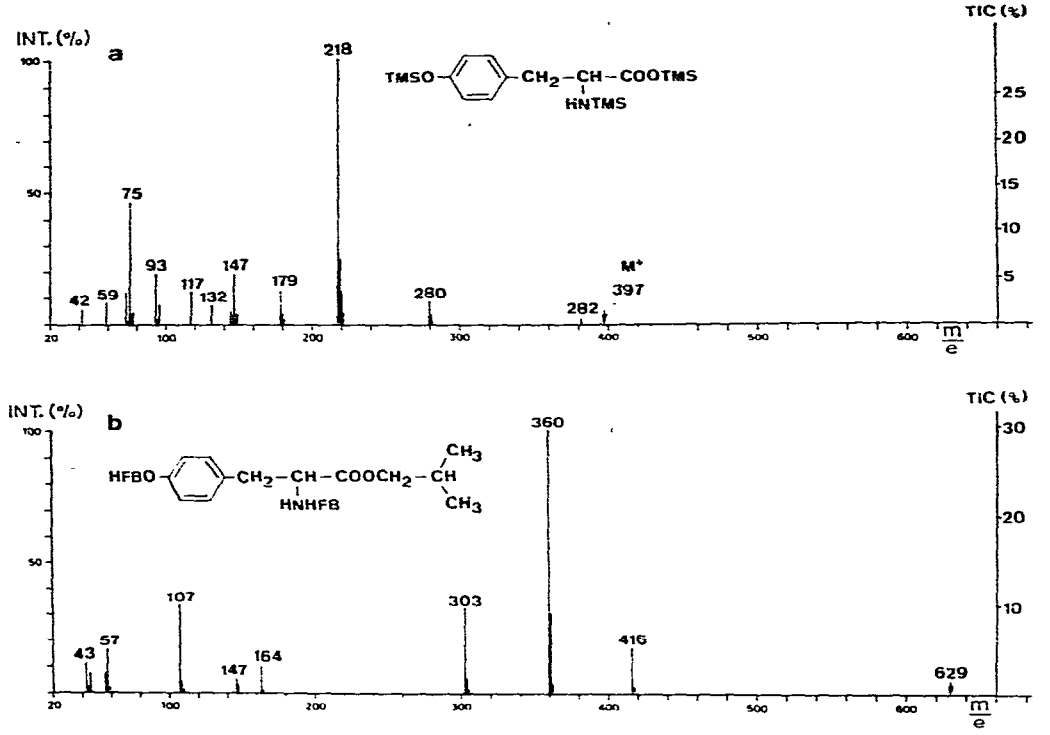
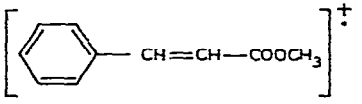
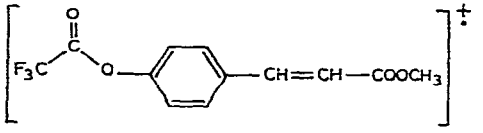


Fig. 2. Mass spectra of tyrosine: (a) N-,O-bis(trimethylsilyl) trimethylsilyl ester; (b) N-,O-bis(heptafluorobutyryl) isobutyl ester.

mass spectra of N-, (O-)-trifluoroacetyl methyl ester derivatives show very intensive predominant signals at *m/e* 162, corresponding to the fragment ion



for phenylalanine and at *m/e* 274, corresponding to the fragment ion



for tyrosine. The derivatization described by Donike[4] and Schwarz and Michael[5], using the N-trifluoroacetyl-O-trimethylsilylation, appeared to fulfil our requirements for selectivity, but owing to multiple peak formation (N-trifluoroacetyl-N-trimethylsilyl-O-trimethylsilylation as the side-reaction), difficulties were experienced with re-

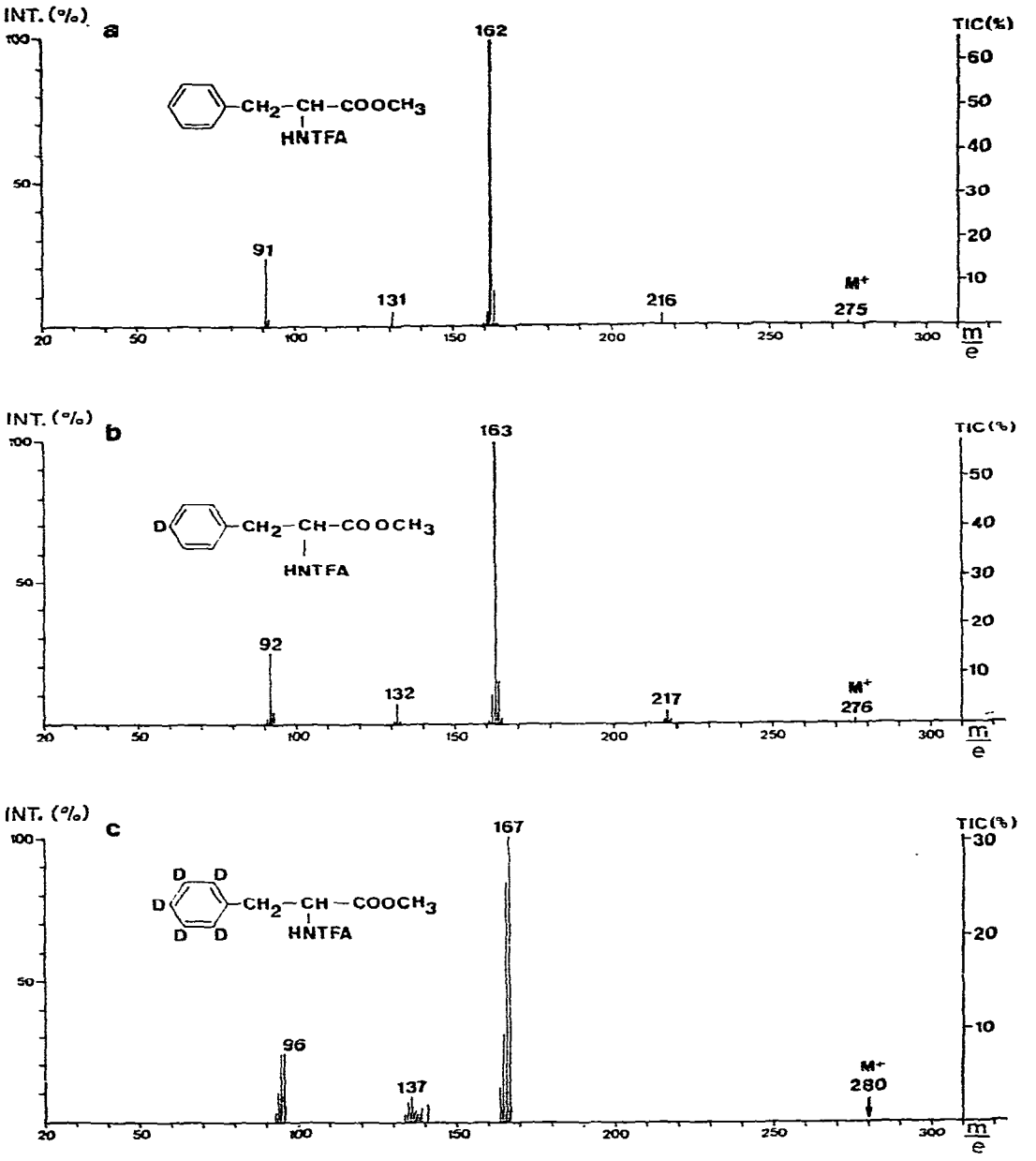


Fig. 3. Mass spectra of N-trifluoroacetyl methyl ester of: (a) phenylalanine-d_0; (b) phenylalanine-d_1 (internal standard); (c) phenylalanine-d_5 (loading material).

producibility. Subsequent modification as proposed by Donike[6] did not, in our hands, overcome these problems.

The trimethylsilyl derivatives have been widely investigated and chosen by many researchers[7–10] for the gas chromatographic analysis of amino acids. We check-

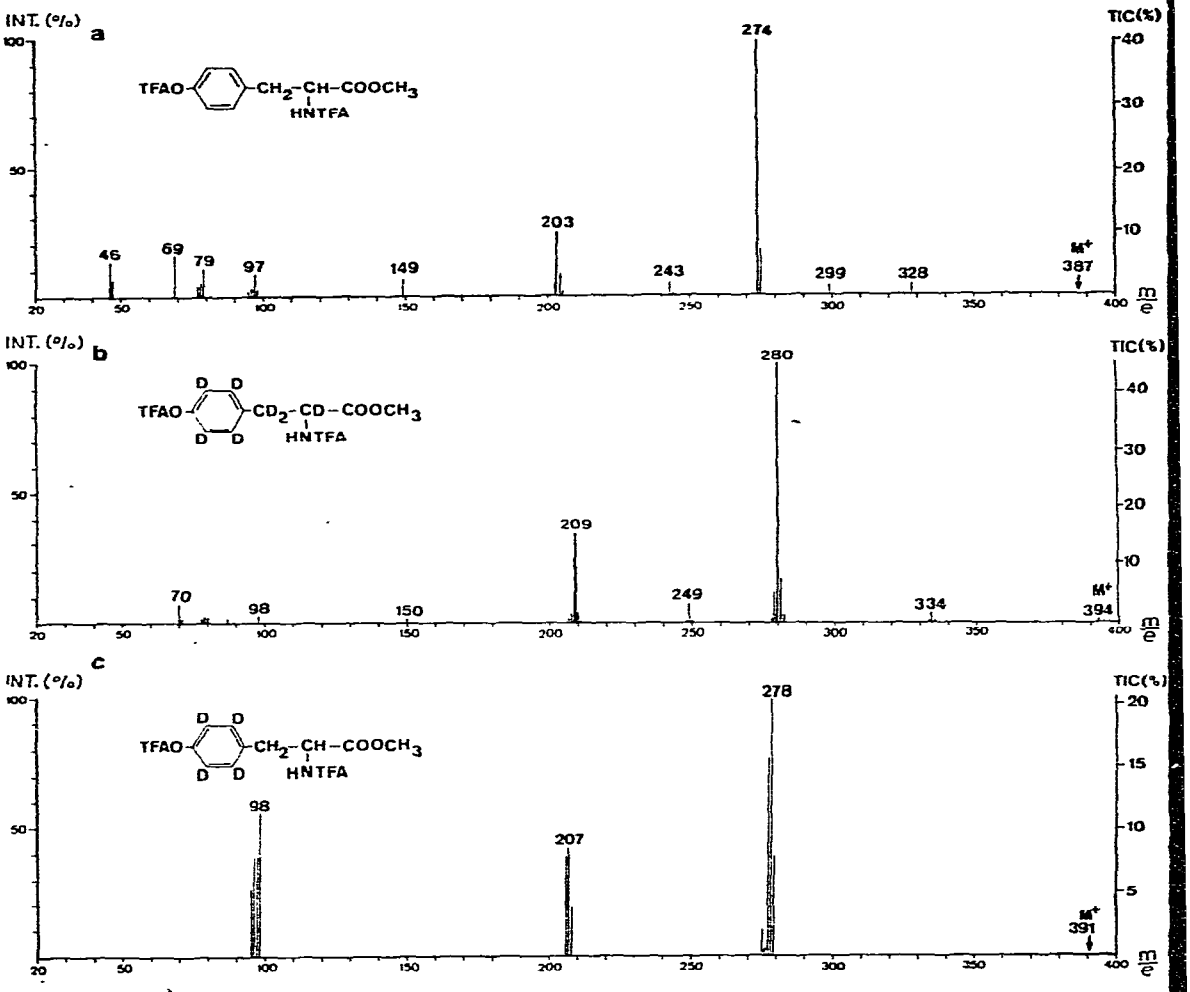
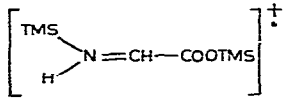


Fig. 4. Mass spectra of N-,O-bis(trifluoroacetyl) methyl ester of: (a) tyrosine-d_0; (b) tyrosine-d_7 (internal standard); (c) tyrosine-d_4 (originating from the loading material, see Fig. 3c).

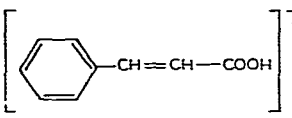
ed these derivatives and discovered that they have two disadvantages which made them unusuable as derivatives for the SIM determination. Firstly, the base signal in both instances (Fig. 1a for phenylalanine and Fig. 2a for tyrosine) is at *m/e* 218:



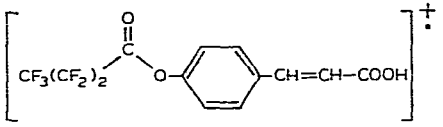
which is a characteristic fragment for all α-amino acids and, therefore, is non-specific for phenylalanine or tyrosine. The other signals are not very suitable for SIM owing to their relatively low intensities. Secondly, the signals representing the natural iso-

topes of silicon would interfere with the quantitative estimation of deuterated standards.

The N-, (O-)-heptafluorobutyryl isobutyl esters[11–13] have been found to be the most suitable derivatives of aromatic amino acids. The mass spectra obtained are excellent (see Fig. 1b and 2b) and show the main signals at *m/e* 148:



for phenylalanine and at *m/e* 360:



for tyrosine. For these and similar derivatives the esterification according to Fischer is usually employed. However, this technique is not recommended for deuterated compounds because exchange of deuterium may take place under the acidic conditions used. Therefore, the esterification was performed with diazomethane.

The SIM chromatograms shown in Fig. 5 represent a typical plasma sample from a healthy subject loaded with phenylalanine-d_5. The first peaks, at *m/e* 162 and

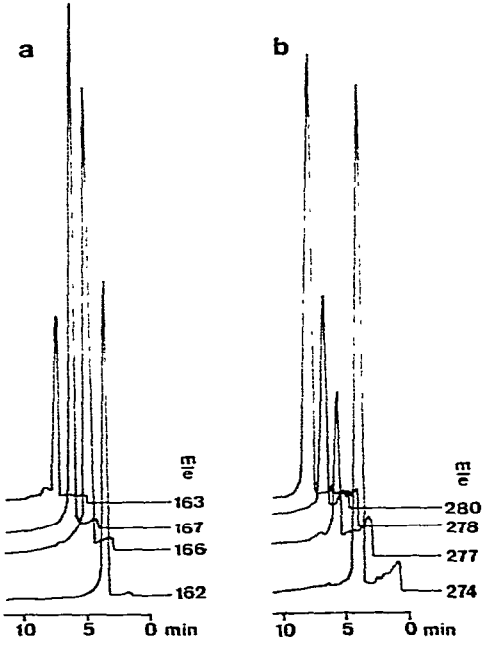


Fig. 5. SIM chromatograms of (a) phenylalanine and (b) tyrosine.

m/e 274 for phenylalanine-d_0 and tyrosine-d_0, respectively, represent the undeuterated compounds. The last peaks, at *m/e* 163 for phenylalanine-d_1 and at *m/e* 280 for tyrosine-d_7, show the internal standards. The peaks between them are derived from the administered phenylalanine-d_5 and -d_4 (*m/e* 167 and 166) and from tyrosine-d_4 and -d_3 (*m/e* 278 and 277); the latter is formed from the labelled phenylalanine by the phenylalanine-4-monooxygenase system.

One plasma sample was analyzed 12 times in order to estimate the reproducibility of the entire procedure (Table I).

TABLE I

STANDARD DEVIATION (*S*) AND COEFFICIENT OF VARIATION (*CV*) OF THE DETERMINATION OF DEUTERATED AND NON-DEUTERATED PHENYLALANINE AND TYROSINE ($n = 12$)

Parameter	$\bar{x} \pm S$	*CV (%)*
Total phenylalanine (mg per 100 ml of plasma)	11.36 ± 0.18	1.58
Deuterated phenylalanine (mg per 100 ml of plasma)	9.44 ± 0.15	1.59
Deuteration (%)	83.13 ± 0.59	0.71
Total tyrosine (mg per 100 ml of plasma)	2.38 ± 0.07	2.94
Deuterated tyrosine (mg per 100 ml of plasma)	1.05 ± 0.04	3.81
Deuteration (%)	44.13 ± 0.44	0.997

Amounts as small as approximately 2.5 ng/ml in plasma could be measured with this method.

The use of loading material labelled with a stable isotope seems to be very useful for the determination of the enzymatic activity of phenylalanine-4-monooxygenase in humans and is also safe for the subjects in comparison with radioactive tracer experiments. Labelling with ^{13}C would be even more suitable than deuteration, as the isotopic effect of ^{13}C is extremely small and there is less danger of exchange in the body. The natural abundance of ^{13}C, however, is much higher than that of ^{2}H and, therefore, decreases the sensitivity of the method. In addition, the deuterated compounds are less expensive.

The technique described here could probably be used not only for phenylalanine-4-monooxygenase, but also analogously for other enzymatic assays when using the appropriate substrate (as has been shown for the tyrosine-3-monooxygenase, E.C. 1.14.16.2)[31].

We have applied the method successfully to the differentiation of phenylketonuric and hyperphenylalaninaemic patients, as described elsewhere[32].

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