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Phenylalanine in whole blood and plasma — a candidate reference method

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ABSTRACT

A method for the quantification of phenylalanine in whole blood and plasma by isotope dilution gas chromatography–mass spectrometry is presented. The use of an uncommon derivative allows a simple extraction procedure and the most basic mass spectrometer. The relative standard deviation was found to be 1.3% within-batch and 2.1% between-batch under optimum conditions, and the detection limit was found to be 7 pmol injected. The ruggedness of the procedure and sample handling conditions were also examined.

INTRODUCTION

The demand for strictly validated reference methods in clinical chemistry arises for several reasons. The need for reference data on the concentrations of many compounds in biological matrices and assigment of values to reference materials used to assess the performance of routine methods are two such reasons. Furthermore, many countries are moving towards systems of laboratory accreditation. Therefore it will require laboratory procedures to be documented fully with regard to analytical performance.

The inherent accuracy of isotope dilution mass spectrometry (IDMS) has lead to the proposal of its use as a reference method in clinical chemistry [1,2]. We present an isotope dilution method for phenylalanine in plasma and whole blood and characterise the analytical performance of that procedure.

EXPERIMENTAL

Reagents

Trifluoroacetic acid (TFAAc) and trifluoroacetic anhydride (TFAAn) were obtained from Sigma (Poole, U.K.) and ethyl acetate and 98% ethanol were Analar grade obtained from BDH (Dagenham, U.K.). Unlabelled phenylalanine (99+%) was from Fluka Chemicals (Glossup, U.K.). All chemicals were used

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without further purification. All water used was distilled, deionised and redistilled from glass.

A 20 mmol l^{-1} stock standard solution of phenylalanine was prepared and working standards were prepared from this stock by dilution. A solution of the internal standard of approximately 40 mmol l^{-1} was prepared and used without further dilution. Both were stored at −20°C in suitable aliquots, being thawed as required.

Gas chromatograph–mass spectrometer

The system available comprised an LKB 9000S single-focussing magnetic sector instrument. The machine was modified to accept a Carlo Erba Fractovap 2150 Series gas chromatograph. The columns used were 2 m × 3 mm glass packed with either 1% or 3% OV1 on Diatomite CLQ, 100–120 mesh (supplied ready coated by J.J.'s (Chromatography), Kings Lynn, U.K.). Several electronic modules from the LKB were replaced with units more amenable to control by the data system, namely the ion source HT supply, the magnet control unit and the electron multiplier preamplifier. All were replaced by units from a VG 70-70. The source conditions were: electron-impact (EI), 70 eV; trap current, 60 μA; source temperature, 250°C. All quantitative data were aquired by selected-ion monitoring (SIM) using voltage switching controlled by the data system. The mass spectrometer resolution was set to 255 (10% valley definition).

The data system comprised a Teknivent Model 802 data acquisition interface (Teknivent, St. Louis, MO, U.S.A.), controlled by a Compaq Deskpro 286 microcomputer (Compaq Computer, Houston, TX, U.S.A.) running Teknivent Vector One Software.

Characterisation of pipettes

For the most accurate work, weighed portions of internal standard solution would be added to sample or standard solutions. In clinical laboratories, however, it is a more common practice to express results in terms of molarity or its subunits. It was felt therefore, that for this preliminary work, volumetric micropipettes would be used for these operations once the delivery of material from them had been assessed for both accuracy and precision.

Selection of derivative and extraction procedure

Many derivatives have been used for gas chromatography (GC) of amino acids, and most of these have been used for gas chromatography–mass spectrometry (GC–MS). Published EI reference spectra were examined where available, and several derivatives were made where spectra were unavailable. The majority of spectra of phenylalanine derivatives examined showed an absent or very small molecular ion under EI conditions. One exception was the 2-trifluoromethyloxazolinone (TFMO) derivative. The spectra of both [$^1H_5$] phenylalanine and [$^2H_5$] phenylalanine as their TFMO derivatives are shown in Fig. 1. An abundant

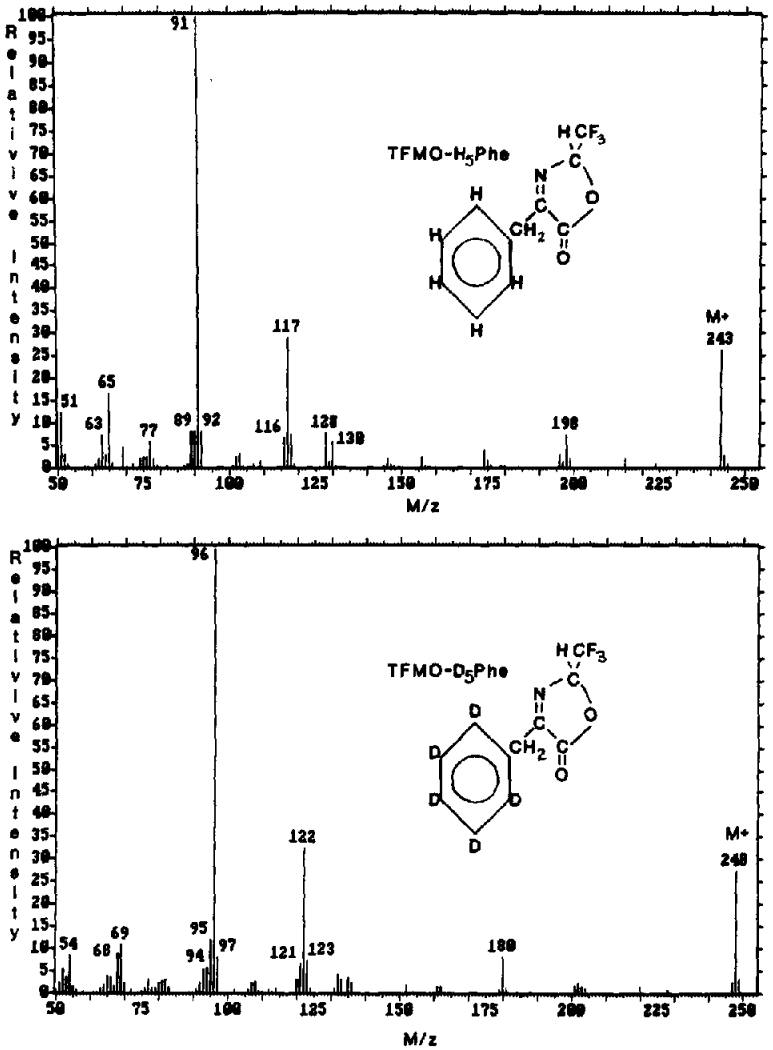

Fig. 1. EI mass spectra (70 eV) of the TFMO derivatives of pure standard phenylalanine (upper), and its pentadeutero analogue (lower) used as the internal standard. An abundant molecular ion can be seen in both cases.

molecular ion is seen in both spectra. The derivative was simply formed in a one-step process as described by Grahl-Nielsen and Movik [3]. The chromatograms of Grahl-Nielsen and Movik showed no interfering peaks using GC alone. It was decided on this evidence that ethanol extraction would be a viable method to use as the extra dimension of SIM would increase selectivity.

Equilibration of sample with internal standard

It has been recommended that for IDMS the internal standard should be added to the sample in a small volume (less than 2%, v/v) and allowed to equilibrate for several hours [4] to minimise the chances of disturbance to the matrix. For this work the sample size chosen was 1 ml, and the internal standard volume was 25 μl.

Equilibration experiments were performed using whole blood, whole blood hemolysed by repeated freeze–thaw cycles, plasma and plasma with white cells still in suspension. The whole and lysed blood experiments were performed at room temperature, 4°C and at room temperature with added Trasylol (Bayer U.K., Newbury, U.K.), a protease inhibitor.

Linearity of calibration and effects of matrix

Standards were prepared at concentrations of 0, 400, 800, 1200, 1600 and 2000 μmol l^{-1} and carried through the whole procedure. Additionally, whole blood was supplemented (in a small volume) with phenylalanine. The amounts added were equivalent to 200, 1000 and 2000 μmol l^{-1}. These samples and the original blood sample were allowed to equilibrate at 4°C for 3 h and analysed in duplicate to assess the effect of the sample matrix on the calibration graph.

Precision studies

As large volumes of material were required, a unit of out of date fresh frozen plasma was obtained from the blood bank. This material was supplemented with phenylalanine at different levels, allowed to equilibrate at 4°C for 3 h and then frozen in suitable aliquots for the studies. The data for the between-batch precision was gathered over a period of two months. One series of samples for within-batch precision studies were injected twice in the same run, and the results were subjected to one-way analysis of variance.

Ruggedness test

A fractional factorial design to test for ruggedness was devised. The factors that were included were variation in the time and temperature of derivatisation, amount of internal standard added, equilibration time with internal standard, drying time during solvent removal and volume of reagent used for derivatisation. The time/temperature and drying time were included as deuterium-labelled compounds tend to be slightly more volatile than their hydrogen analogues, possibly introducing preferential losses in these processes. Full details of the perturbations from the final procedure used are shown in Table V.

Final procedure used for analysis

The following procedure for the analysis of blood and plasma was adopted.

1. Samples should be freshly drawn heparinised blood or plasma obtained from fresh heparinised blood. Samples should be kept at 4°C and analysed within a few hours or frozen at −20°C if this is not possible.

2. Thaw the standard and internal standard solutions and bring to room temperature.

3. Pipette into 2-ml polystyrene tubes either 1 ml of water (as a blank), 1 ml of 2000 μml l^{-1} phenylalanine standard or 1 ml of sample.

4. Add to each tube 25 μl of internal standard solution, mix and cap the tube. Allow to equilibrate at 4°C for 3 h with occasional gentle agitation.

5. Transfer 100 μl from each tube to a clean 2-ml polystyrene tube and add 900 μl of ethanol while mixing vigorously (rotary mixer). Allow to stand for 2 min, then centrifuge for 5 min at 2500 *g*. Remove the supernatant to a 2-ml reaction vial and dry down with a stream of nitrogen at 100°C.

6. To all tubes add 200 μl of a 1:1 mixture of TFAAc–TFAAn. Mix and cap tightly using a PTFE-coated liner in the cap. Place on a hotplate at 130°C for 15 min.

7. Allow the tubes to cool and remove the reagents in a stream of nitrogen *without warming*.

8. Redissolve the residue in 100 μl of ethyl acetate. Inject 2–3 μl into the GC–MS system and record the ion profiles at integer masses 243 and 248.

RESULTS AND DISCUSSION

Precision of pipetting and relative volumes

Using glass micropipettes, the mean delivery volumes for plasma, whole and lysed blood all differ significantly at the 95% confidence level from that of water. Correction factors were applied to results for these materials of 1.004, 1.010 and 1.013, respectively, to obtain the correct result for calibration with aqueous standards. For addition of internal standard to standards and samples the overall calculated relative standard deviation (R.S.D.) was found to be 0.3%.

Interferences

No peaks were seen in sample chromatograms that would interfere with the quantitation of the peak areas or heights. Fig. 2 shows the selected-ion profiles obtained from a whole blood sample taken through the whole procedure for masses 243 (analyte) and 248 (internal standard). It can also be seen that late-eluting peaks are not present to interfere with subsequent injections.

Sample equilibration

A preliminary experiment with whole blood showed that the equilibration of internal standard with the sample matrix was probably complete within 2 or 3 h, but a slight increase in apparent concentration was obtained after 4 h. To clarify this a more comprehensive experiment was performed over 24 h. The results are shown in Fig. 3. The small differences seen in measured phenylalanine for plasma and plasma plus white cells are not significant when the precision of the assay at this level is considered. The slow upward trend in measured phenylalanine seen in

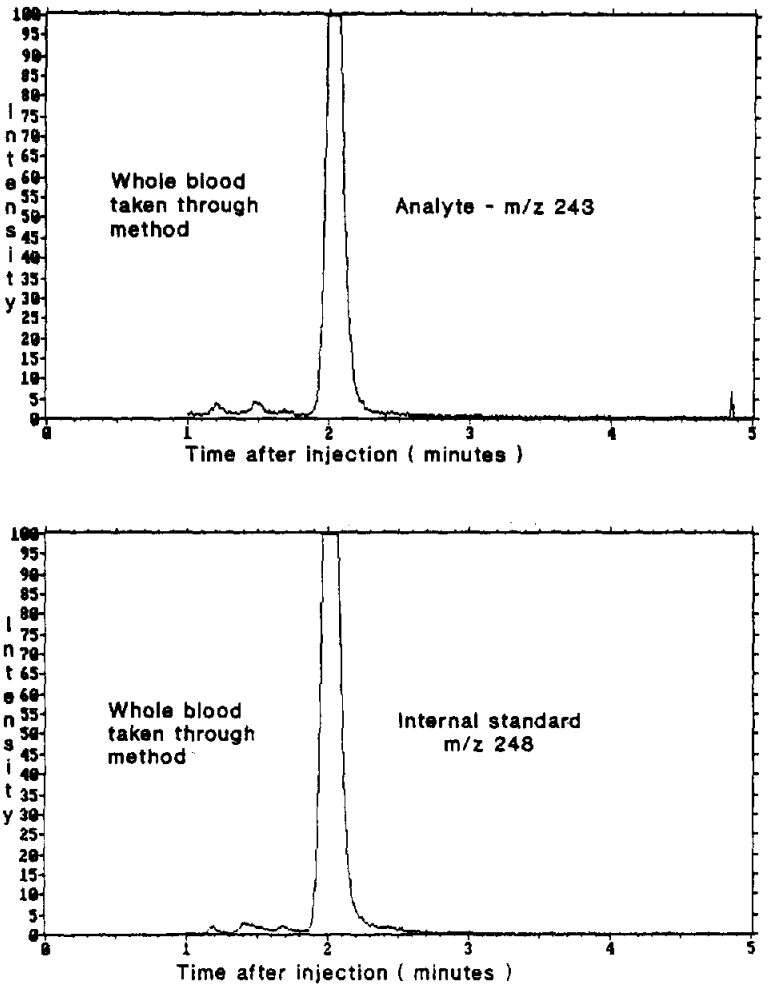

Fig. 2. Selected ion profiles of *m/z* 243 (upper) and *m/z* 248 (lower) used to monitor the TFMO derivatives of phenylalanine and the internal standard, respectively. The retention times are 2.03 min for phenylalanine and 1.98 min for the internal standard, illustrating the deuterium isotope effect. Traces are shown magnified approximately five times to show baseline detail. GC conditions are described in the text.

whole and lysed blood is more striking, being most obvious with the lysed specimen. It was shown that the concentration measured did not depend upon the time after the addition of internal standard, but upon the time the sample had stood at room temperature before analysis.

The addition of a protease inhibitor Trasylol (Bayer U.K., 200 kI.U. ml^{-1} of sample) and standing at 4°C were examined in an attempt to prevent this effect. From Fig. 4 it can be seen that refrigeration at 4°C is effective in preventing the rise in phenylalanine concentration in the sample for at least two days. The addition of Trasylol appeared to have no significant effect. During the course of

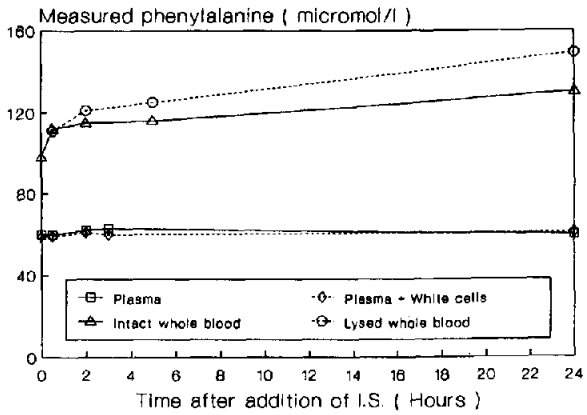

Fig. 3. Effect of equilibration time on measured phenylalanine concentration at room temperature for different analytical materials. Conditions are described under Experimental.

these experiments, changes of up to 8 μmol l^{-1} in 3 h were observed in some samples. It is therefore recommended that equilibration of the sample with internal standard should be performed at 4°C to prevent possible systematic error.

Linearity and matrix effects

A calibration curve constructed from triplicate standards indicated that the method was linear over the range studied, 0–2000 μmol l^{-1}. Linear regression of response ratio m/z 243/m/z 248 on phenylalanine concentration yielded a correlation coefficient of 0.9999 and the equation of the line was $y = 0.00109x + 0.00348$.

To test for possible matrix effects, recovery experiments were performed at three levels. Whole intact blood was considered to be the "worst case", where the intact cells present were most likely to cause problems. Samples were prepared and analysed in duplicate by the proposed procedure. The results are shown in Table I; each value is the mean of two determinations.

Precision studies

The precision of the procedure within-batch was considered at seven concentration levels and between-batch at six levels. The results from these experiments are tabulated in Tables II and III. The standard deviation (S.D.) and R.S.D. results calculated for both peak-area and peak-height measurements are presented and depicted graphically in Fig. 5. These data indicate that more consistant results are obtained from peak heights than from peak areas. This is surprising, but has been reported by other workers using the same model of mass spectrometer [5,6]. The graphs indicate that the peak-height data follow the accepted model for S.D. and R.S.D. variation with concentration.

The detection limit for the procedure calculated from three standard devia-

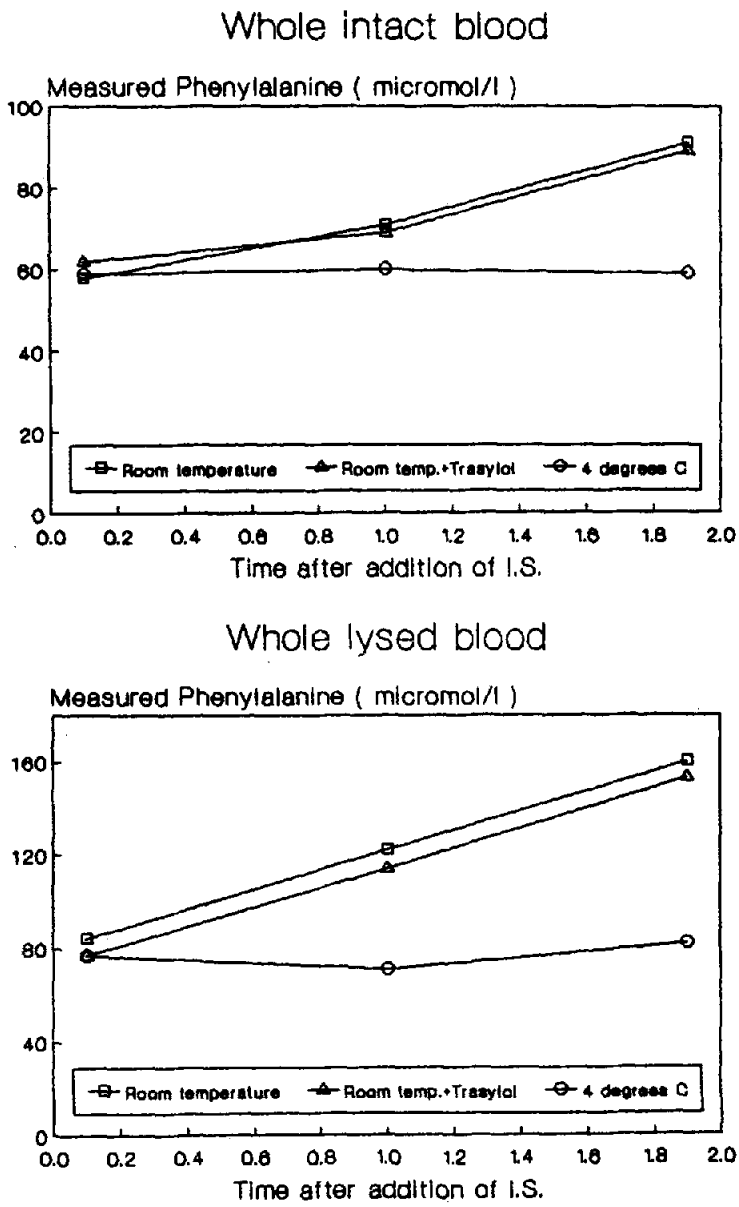

Fig. 4. Results of experiments to remove *in vitro* formation of phenylalanine. Conditions are described in the text.

tions of the blank response ratio is 3.3 μmol l^{-1}, corresponding to approximately 7 pmol injected.

To examine the source of the imprecision observed, duplicate injections of one series of samples were made. One-way ANOVA, performed on the peak-height

TABLE I

RESULTS OF RECOVERY EXPERIMENT

Data obtained using the final procedure for analysis.

Sample	Phenylalanine added	Result	Phenylalanine found	Recovery (%)
1	0	48.7	Base sample	
2	200	245.1	196.4	98
3	1000	1079.3	1030.6	103
4	2000	2032.3	1983.6	99

TABLE II

WITHIN-BATCH PRECISION DATA

Data obtained using the final procedure for analysis.

n	Peak height			Peak area		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
6	0	1.1	–	–	–	–
10	49.9	1.7	3.4	54.9	3.9	7.1
10	194.2	4.5	2.3	199.6	9.7	4.9
10	387.3	6.8	1.8	403.4	8.6	2.1
10	832.1	10.9	1.3	820.5	20.8	2.5
10	931.4	12.8	1.4	956.6	8.3	0.9
10	1207.9	17.2	1.4	1214.8	20.1	1.7
10	1671.2	28.2	1.7	1668.7	12.6	0.8

TABLE III

BETWEEN-BATCH PRECISION DATA

Data obtained using the final procedure for analysis.

n	Peak height			Peak area		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
8	58.3	9.3	16.0	61.7	11.1	18.0
8	180.5	7.7	4.3	183.4	7.7	4.2
7	402.7	13.7	3.4	407.5	16.4	4.0
8	823.4	17.4	2.1	833.2	17.2	2.1
8	1221.0	29.6	2.4	1231.9	22.3	1.8
8	1694.6	45.7	2.7	1695.6	51.6	3.0

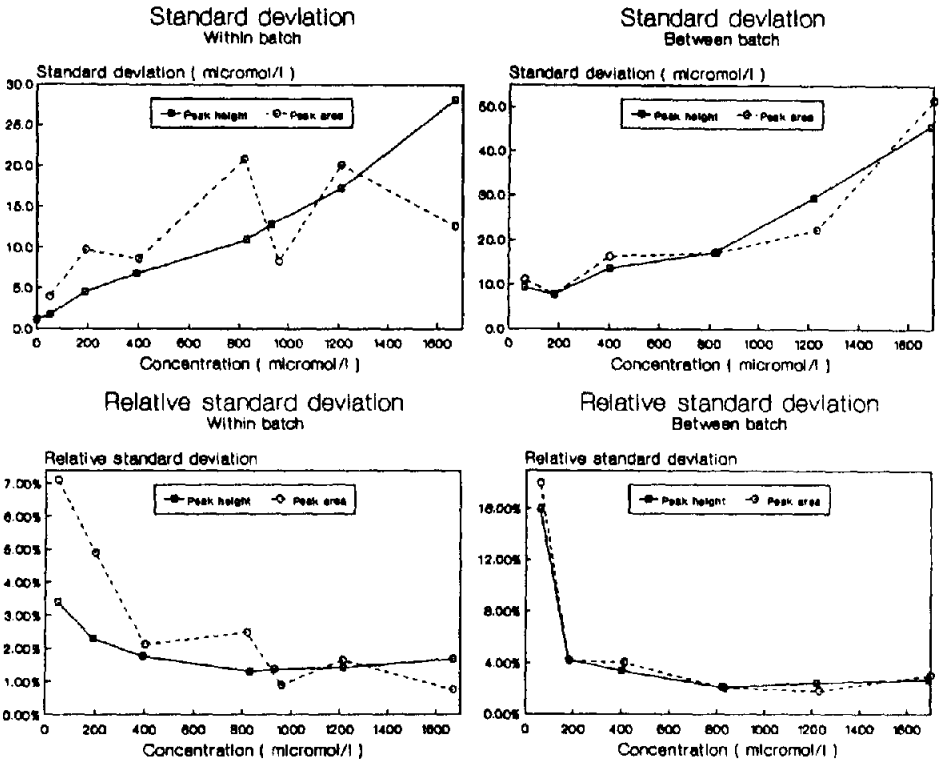

Fig. 5. Precision profiles for within-batch (left) and between-batch (right) precision, expressed as S.D. (upper) and R.S.D. (lower). Details are given in Tables II and III. Data obtained using the final procedure for analysis.

TABLE IV

RESULTS FROM ANOVA OF DUPLICATE INJECTIONS

Experimental conditions are described in the text.

Source of variance	Sum of squares	Degrees of freedom	Mean square	$F_{9,10}$
Between-sample	1997.4245	9	221.94	1.222
Within-sample	1802.905	10	181.6650	
Total	3814.0895	19		

One-tailed F_{critical} (95%) = 3.020

TABLE V

EFFECTS OF PERTURBATIONS IN THE FRACTIONAL FACTORIAL EXPERIMENT

Explanation of perturbations: derivatisation time, the sample derivatisation time was extended to 20 min; derivatisation temperature, the temperature of derivatisation was increased to 140°C; I.S. quantity, the amount of internal standard added was doubled, the result being adjusted accordingly; I.S. equilibration time, after addition of the internal standard, the sample was precipitated with ethanol within 3 min; dry time, when removing the derivatisation reagents, after the last traces of solvent were gone, the sample was blown with nitrogen for a further 30 s; volume of reagent, the volume of reagent used for derivatisation was reduced by half.

Factor	$\|t\|^a$
Derivatisation time	2.44
Derivatisation temperature	1.96
I.S. quantity	1.29
I.S. equilibration time	4.75
Drying time	5.20
Volume of reagent	4.43

[a] The critical value of t for the experiment was 2.45.

data, results, shown in Table IV, show that the variance between samples is not significantly different from the variance between injections, suggesting that instrumental factors are largely responsible for the unexpectedly poor precision.

Test of ruggedness

The results of the fractional factorial experiment are shown in Table V. The factors shown to be significant at the 95% confidence level were the equilibration time with the internal standard, the drying time and the volume of reagent. Interactions could not be studied from the results of this experiment as three fractors were significant.

In retrospect the perturbations in the equilibration time and the volume of derivatising reagent were rather excessive and not representative of the deviations that might occur in normal use. Considering the previous experiments on sample equilibration with internal standard, small deviations in time allowed of say a few minutes should have no observable effect on the results. However, the extended blowing dry was a realistic deviation. This result emphasises both the volatility of this particular derivative and the differential volativity of deuterium/hydrogen analogues. This stage of sample preparation should therefore be a closely monitored step in the analysis, possibly to the extent that samples are individually dried after derivatisation, rather than being dried in a batch.

CONCLUSIONS

An IDMS method is presented for the measurement of phenylalanine in blood and plasma. Linearity was demonstrated up to 2000 μmol l^{-1}, and the detection limit was found to be 3.3 μmol l^{-1}. The relative precision of the procedure over the range studied was found to be 1.3–3.4% within-batch and 2.1–16% between-batch. Instrumental contributions to the imprecision were shown to be considerable, and reduction of the R.S.D. at all levels would be expected if a different (*i.e.* more modern) instrument were used. The importance of sample refrigeration both before and during the initial stages of preparation was demonstrated.

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