tain the applicability of tandem MS methods for the determination of the primary structures of glycans.


Use of continuous-flow combustion MS in studies of human metabolism

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Introduction

Over the last 15 years the study of human metabolism has been revolutionized by the application of the use of stable-isotope tracers. Three factors have been of major importance in developing this field of research: first, the recognition that stable isotopes, because of their non-radioactive nature, offer the possibility of following human metabolism in circumstances in which the use of radioactive tracers would be unacceptable (e.g. in children, repeat studies in adults, tissue incorporation measurements, etc.); secondly, the growth of companies supplying tracers labelled with stable isotopes of carbon, O2, H2 and N2; and thirdly, the development of a range of technically sophisticated but user-friendly mass spectrometers with which to measure the extent of labelling of the wide range of biological molecules.

The growth in the application of the techniques and the resulting benefits has been dramatic. For example, the application of the doubly labelled water technique has revolutionized studies of energy balance [1], and our understanding of intermediary human metabolism of fat, protein and carbohydrates is growing apace [2]. Nevertheless, there are currently many studies which we can conceive in principle but which are difficult in practice, partly because of...
the lack of availability of particular labelled compounds, which is often solved simply by paying enough to have the compounds synthesized. A more difficult problem, in our view, is the lack of suitable instrumentation. These problems can be easily understood by a consideration of the strengths and weaknesses of the two major techniques used at present, i.e. isotope ratio mass spectrometry (IRMS) of fixed gases such as CO₂, N₂ and H₂ on the one hand, and capillary gas chromatography–mass spectrometry (GC–MS) on the other.

There are also differences in how the results from these two techniques are normally presented. The IRMS results are expressed as 'δ', i.e. parts per thousand differences from defined standards, whereas GC–MS gives isotope ratios directly. For metabolic tracer studies the required parameter is the mol% excess (MPE) of the tracer, which is obtained directly from the GC–MS analysis, but indirectly from the IRMS result. As a rule of thumb, an increase of 1‰ is equivalent to 0.001 MPE for C, 0.0003 MPE for N₂, 0.0002 MPE for O₂ and 0.00002 MPE for H₂.

IRMS is a technique which is particularly useful for the very precise measurement of analytes which contain stable isotope tracers and which can be converted into fixed gases. Thus, for conventional off-line IRMS, biological samples which can be converted into N₂, CO₂, CO or H₂ and which retain the tracer isotope during the conversion process can be analysed with good precision (±0.3‰) [3] so long as the sample is present in sufficient amounts (≤1 μmol) [4]. In a few particular cases, e.g. using CO₂-trapping devices, the sensitivity can be extended to the nmol/pmol range [5]. Thus the method is particularly good for measuring the rate of appearance of the oxidation products of fats, carbohydrates and amino acids as CO₂ in the breath or the rate of appearance of amino acids into proteins from which the ¹³C-labelled carboxy group may be freed after acid hydrolysis, separation, purification and reaction with ninhydrin to form CO₂ [6].

With GC–MS, on the other hand, intermediary metabolites such as sugars, short-chain organic acids, fatty acids, amino acids, steroids and purines and pyrimidines may have their tracer labelling measured (more-or-less in the intact state) after the formation of simple volatile adducts by the use of a variety of derivatization agents [7]. After separation by GC, each compo-
tivity, accuracy and precision. For H₂, the major problem has been the lack of a mass-spectrometer system capable of high-precision H₂ analysis in an excess of He. For N₂-containing organic compounds the major problem is the high background of N₂ in the air which tends to leak into the analytical gas train, with obvious deleterious results for measurement of isotope ratios. It is also important to ensure complete removal of CO₂ from the N₂ as this interferes with the analysis due to the formation of CO in the ion source.

Practical problems such as those resulting from derivatization agents containing a large number of carbon atoms, e.g. t-butyldimethylsilyl (TBDMS), have to be considered. For example, for most amino acids 2 mol-equivalents (ME) of amino acids have to be combusted to generate 1 ME of N₂. Using TBDMS derivatives for an amino acid such as t-proline, an excess of 34 ME of CO₂ is produced for 1 ME of N₂. This excessive carbon load can lead to temporary overload of the oxidation catalyst, thus resulting in incomplete combustion and formation of CO which cannot be trapped cryogenically and hence will interfere with the measurement of N₂ isotope ratios.

Some of these problems and others such as control of the gas train and data collection and reduction problems are being overcome and, if not completely overcome yet, are at least identified. It therefore seems likely that involvement of GC–MS: for 15N measurements typically only 10–20 pmol of amino acids needs to be injected on to the GC column and for 13C typically only 0.1–1 pmol is required. In contrast, when an elemental analyser is coupled to an IRMS (EA–IRMS), 0.5–5 μmol of starting material (for 15N) and 0.1–0.5 μmol (for 13C) are required. GC–C–IRMS provides analytical precision which is much better than can be obtained by conventional GC–MS: for 15N typically ± 1.0‰ and better can be obtained (N.B. the units refer to measured δ values, not to relative precision).

Examples of the use of GC–C–IRMS for the measurement of labelled proteins and nucleic acids

The utility of the method can be seen in the results obtained from the analysis of porcine plasma albumin after unprimed constant infusion of tracer amounts of [1-13C, 15N]alanine, [1-13C, 15N]leucine and [15N]phenylalanine during a physiological study. The results show the diagnostic potential of 13C/15N-doubly labelled amino acids for studies of tissue protein synthesis and amino acid pools involved (Figure 1). All the amino acids were quantified in the same analytical run. The observed enrichment ratios of 15N/13C found in plasma protein were on average 1:3.18 and 1:2.29 for alanine and leucine respectively. In the absence of any 13C cross-over (δ13C-values for phenylalanine remained at baseline level during the course of the study), this observation clearly indicates that alanine and leucine underwent transamination before their incorporation into plasma protein.

The methods have the potential to make possible a new area of biological tracer work, one that holds out the promise of adding a much needed quantitative aspect to the study of gene expression. This is the measurement of RNA turnover. In recent experiments we have shown that, after an intravenous infusion of [1,313C]orotic acid (10 μmol/h per 250 g rat) over 90 min, total RNA in liver was labelled to the
Figure I

Labelling of porcine albumin sampled during the primed constant infusion of a mixture of stable-isotope-labelled amino acids, i.e. \( ^{1-13}C,^{15}N \)alanine, \( ^{1-13}C,^{14}N \)leucine and \( ^{15}N \)phenylalanine

At each time point analysis was carried out for each amino acid in a single analytical run. Albumin was hydrolysed with 6 M HCl and the amino acids derivatized using TBDMS.

extent of 0.03 atom% excess. We intend to exploit the sensitivity of the methodology to measure incorporation of tracer into mRNAs for specific abundant proteins identified by Northern blotting. We plan to study the synthetic rate of individual proteins after immunoprecipitation. Together these techniques should allow us to markedly expand our horizons of what can be measured in studies of human metabolism in physiological and pathophysiological circumstances.

Development of methods to measure collagen synthesis in adult humans

Collagen is the most abundant single protein in the human body, but we know very little about its turnover and the physiological control of this and possible pathophysiological derangements. When we tried to develop methods using preparative GC with off-line EA–IRMS to measure labelling of isolated proline and hydroxyproline from collagen, we immediately discovered problems in reproducibly separating them in large enough amounts, and found that contamination with other amino acids made the results difficult to accept. These problems have been overcome with GC–C–IRMS. We carried out studies in patients about to undergo the removal of the head and neck of femur in preparation for the insertion of an artificial hip joint: a large bolus dose of labelled L-proline was administered after 6 h of continuous infusion of L-[\(^{15}N\)]alanine. The proline was labelled with \(^{15}N\) in one set of studies and with \(^{13}C\) in the other. The usefulness of proline as a tracer lies in the phenomenon of post-translational hydroxylation of protein-bound proline in collagen. By definition, all protein-bound hydroxyproline is in collagen and therefore analysis of the change of labelling in protein-bound hydroxyproline, by comparison with the extent of the labelling in the free proline pool, will provide a value for the rate of collagen protein synthesis. The relative advantages and disadvantages of using \(^{15}N\) and \(^{13}C\) as a tracer under these circumstances are well illustrated by the results. When \([^{15}N]\)proline was used it was possible to detect changes in labelling of proline and hydroxyproline of \(\sim 0.002\)–\(0.003\) atom% with an average precision of 0.0005 atom%. A major advantage of using \(^{15}N\) as a tracer for GC–C–IRMS is that, so long as a derivatization agent containing no N is used, there is no dilution of the tracer/tracer ratio during analysis.

This is certainly not the case with \(^{13}C\)-labelled substances. For example, given a similar rate of bone collagen synthesis, then using similar amounts of \(^{13}C\)-labelled proline and a TBDMS derivative which adds 12 carbons to proline and 18 to hydroxyproline, there is a substantial amount of dilution; thus changes in the labelling of collagen-derived proline and hydroxyproline were small, i.e. values of 0.5–2‰ were detected with a precision of 0.15‰ on average. When converted to MPE after correcting for the carbon added by derivatization it was possible to detect changes in proline and hydroxyproline enrichment of between 0.012 and 0.06 MPE. The precision did not change.

One unusual feature of the results was that the labelling of hydroxyproline exceeded that of proline by 26%, an apparent violation of the expected product–precursor relationship (Figure 2). This could, however, be reconciled with a model in which a rapidly turning over pool of collagen was hydroxylated, which fits with current models of collagen processing.

GC–C–IRMS for \(^3\)H-labelled compounds

Development of GC–IRMS for measuring \(^3\)H/H ratios has followed the production of a novel mass spectrometer capable of analysing H\(_2\) gas in continuous-flow mode [18]. He is the carrier gas of choice for continuous-flow systems, and, in order to avoid spillage of He\(^{+}\) ions into the HD collector, a radical redesign of the mass spectrometer was necessary. The redesigned mass spectrometer gives adequate dispersion of the He and
Relationship between the labelling of bone-collagen-derived hydroxyproline and proline with $^{13}$C after flooding-dose infusion of [1-$^{13}$C]proline (30 atom%) in patients undergoing resection of head and neck of femur for hip replacement

![Figure 2](image)

$^2$H/$^1$H molecular ions to give a precision of ±2%o for $^2$H/$^1$H. One of the major advantages of the new analyser design is that it may also be used to measure isotope ratios of other permanent gases, e.g. $^2$N, CO or CO$_2$, without requiring any change to the inlet configuration.

The problems associated with on-line methods for analysis of $^2$H-labelled compounds are primarily those of sample memory and incomplete conversion. A method has been developed for reduction of water to H$_2$ and CO in a specially adapted reduction furnace [19]. On-line reduction by this method has been found to be perfectly adequate for the analysis of nanolitre volumes of water with a precision of ±4%o for H$_2$ and ±0.4%o for O$_2$. The technique is appropriate for the determination of total energy expenditure by the doubly labelled water method, being accurate over a wide range of enrichment.

Subsequently, an improved reduction–pyrolysis technique has been developed for the analysis of H$_2$ in biological fluids (urine and saliva) or volatile organic compounds after their quantitative conversion into H$_2$ and CO. In this case the alumina tube reduction furnace was modified from that used for water by being packed with nickel wire. The carbon coating (necessary for production of CO from water) is regenerated daily or after ~50 injections of carbon-free water. Organic compounds contain sufficient carbon for their reduction so that regeneration is unnecessary. Natural abundance $^2$H/$^1$H isotope ratio values for acetone, ethanol, methanol, ethylbenzene and hexane have been measured with a precision of 2–4%o. When dilution series for ethanol, acetone and ethylbenzene were prepared, with corresponding $^2$H-labelled compounds, highly linear correlations ($r^2 = 0.9999$) between the measured $\delta^2$H and the amount of added label were obtained over a range of 0.075 atom% excess. As for water analysis, only nanolitre quantities of organic compounds enter the reduction furnace after split injection of 0.5 µl. This potentially broadens the application of GC–IRMS techniques to the use of a wide range of $^2$H-labelled tracers in biological compounds. With chromatographic separation before reduction–pyrolysis, the analysis of many components from a single sample would be possible.

**Future developments and outlook**

The on-line conversion of organic compounds into H$_2$ opens up the way to the analysis of a wide variety of cheap deuterated tracer materials. Since the technique would allow analysis of very small amounts of the original analyte, it would be particularly useful in paediatric studies or studies in which heel prick or ear lobe blood sampling confer substantial benefits in reducing the invasive nature of investigations. The single most widely used deuterated material for human metabolic studies is dideuteroglucose and the use of new technology for investigations of glucose turnover would be a major advantage. Other applications would lie in the study of the incorporation of $^2$H from the body water pool into fatty acids and cholesterol but further applications in food and drink authentication and plant and animal biochemistry are readily envisaged.

In order to take full advantage of the possibilities offered by the instrumentation we need to develop a set of derivatization procedures that add the minimum amount of extra atoms of the element of interest, otherwise dilution will limit sensitivity. Some obvious candidates that minimize the introduction of carbon are O-trimethylsilyl, O-propyl and N-trifluoroacetyl. We are currently developing suitable derivatization protocols using some of these for amino acids.

The problem is one we have now for carbon-containing tracers, but it will be even more of a problem for $^3$H, unless tracer materials containing more isotope are synthesized, a relatively expensive approach, but one which is feasible.

One of the prizes to be grasped if the other technical problems can be overcome is that of simultaneous measurement of $^{13}$C and $^{15}$N label-
The use of automated electrospray ionization tandem MS for the diagnosis of inborn errors of metabolism from dried blood spots

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Introduction

The clinical consequences of inborn errors of metabolism become apparent (i) slowly because damage is caused by chronic accumulation of toxic intermediates or (ii) acutely after injury or infection. The latter presentations are due to sudden metabolic decompensation caused by accelerated catabolic flux of intermediates along a metabolic pathway compromised by a disease-specific enzyme defect. The acute symptoms, including a reduced level of consciousness, fits, circulatory collapse and liver dysfunction, fall within the differential diagnosis of many other more common diseases and diagnosis can therefore be difficult. It can be several weeks before the appropriate investigations are instigated and their results received from a specialist centre. Such a delay in diagnosis can be detrimental to the patient resulting in death or severe physical and/or mental disability. Thus there is a need for...