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Application of high-performance liquid chromatography with isotope-ratio mass spectrometry for measuring low levels of enrichment of underivatized materials

Fred P. Abramson*, Gavin E. Black, Paolo Lecchi

Department of Pharmacology, George Washington University School of Medicine and Health Sciences, 2300 I St. N.W., Washington, DC 20037, USA

Abstract

Combining HPLC separations with an isotope-ratio mass spectrometric (IRMS) detection produces a device capable of measuring very low alterations in ^{13}C abundance from analyte species that cannot be volatilized. Examples are presented showing proteins, carbohydrates, and nucleotides that are eluted from varying types of HPLC columns (reversed-phase, normal-phase, ion-exchange and size-exclusion). This wide range of chromatographic methods enables the analysis of compounds never before amenable to IRMS techniques and may lead to the development of many new assays. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

An isotope-ratio mass spectrometry (IRMS) system is a specially-configured MS system optimized for the measurement of small differences in isotopic abundance [1]. Typically, CO_2 derived by combustion of organic molecules is the species being measured. In its basic scheme, the CO_2 is produced off-line and introduced into the IRMS system from a gas reservoir. Barrie et al. [2] coupled a GC system, an in-line combustor (COM), and an IRMS system to examine mixtures of analytes. Since then, several commercial GC–COM–IRMS systems have become available [3]. However, many compounds of biological, medical, and environmental interest are not volatile and have to be derivatized to facilitate GC analysis [4]. Other compounds are not volatilazable at all. To solve the problem of non-volatile analytes, HPLC would be the preferred separation method.

In 1993, Caimi and Brenna [5] coupled HPLC to IRMS. Their approach used a moving wire to transport the non-volatile analytes from the end of the HPLC column to the combustion device. An alternative scheme was described by Teffera et al. [6]; that system used a thermospray nebulizer and a counter-current gas diffusion system to produce a beam of non-volatile particles in a desolvated stream of helium. These particles were converted to CO_2 using a microwave-powered chemical reaction interface (CRI, [7]) with the output passing into the

*Corresponding author. Tel.: +1-202-994-2946; fax: +1-202-994-2870.

E-mail address: phmfpa@gwumc.edu (F.P. Abramson).

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IRMS system. This article describes the performance of our HPLC–CRI–IRMS instrument with a variety of HPLC separation schemes.

2. Experimental

2.1. Instrumentation

The details of the HPLC–CRI–IRMS instrument and its performance have already been published [6]. Basically, the apparatus consists of an HPLC pumping system, a Vestec universal interface, a momentum separator and microwave cavity (Scientific Instrument Services, Ringoes, NJ, USA), and a Finnigan MAT (ThermoQuest, San Jose, CA, USA) Delta S IRMS system with the Isodat data system. The reactant gas was O_2. In each analysis the isotope ratio was standardized either to a compound having a primary value obtained by a reference laboratory or to a second compound with an isotope ratio that we obtained using a primary standard as reference. The isotope-ratio (IR) data are calculated by

$$\delta^{13}C(‰) = 1000\,(\mathrm{IR}_{\mathrm{sample}} - \mathrm{IR}_{\mathrm{std}})/\mathrm{IR}_{\mathrm{std}}$$

Teffera et al. [6] also established the range of sample sizes for which the isotope ratio of a given analyte remains constant. Above this amount, side reactions that are the result of incomplete transformation of analytes generate species that interfere with masses 44 or 45.

2.2. Chromatographic systems

Four different HPLC columns and solvent systems were used. For reversed-phase chromatography we used a 30×2.1 mm PerSeptive Poros R2 column (PE Biosystems, Framingham, MA, USA). For normal-phase chromatography, we used a 250×4.6 mm Asahipak NH2P-50 polymeric amino column (Keystone Scientific, Bellefonte, PA, USA). For ion-exchange chromatography, we used a 75×7.5 mm Bio-Rad Bio-Gel DEAE-5-PW column (Hercules, CA, USA). The size-exclusion chromatography was done with a 250×4.6 mm SynChropak GPC Linear (Keystone Scientific). All mobile phase components were HPLC grade from EM Scientific (Gibbstown, NJ, USA). Several workers have shown that isotopic fractionation occurs in HPLC as is known for GC separations [8,9]. We see this phenomenon also. The Isodat system used to integrate peaks and obtain isotope ratios does not use identical retention times to define peaks, but uses a slope-sensitivity approach. Because the amplifier that detects m/z 45 has a gain of 100× compared to m/z 44, for isotopic abundances near the natural 1% both mass channels generate nearly identical intensities and are integrated properly. In this way, the different elution of ^{13}C-containing species from their ^{12}C-counterparts does not affect the precision of the result.

3. Results and discussion

Most HPLC–MS systems use reversed-phase columns and we have done similar experiments with the IRMS. Fig. 1 shows the analysis of human growth hormone with horse albumin used as an isotopic internal standard. Using such a system, we could show small but significant differences in four growth hormone samples that must reflect the isotopic conditions present when they were synthesized [10]. Such macromolecules can be handled with our interface as easily as small analytes as long as the column is matched to the size of the analyte. Caimi and Brenna first developed the concept of analyzing intact proteins with HPLC interfaced to IRMS [11] and we continue this area of investigation.

For highly polar species, normal-phase chromatography may be preferred. To separate sugars, we use an amino column. Silica-based amino columns were too easily hydrolyzed, leading to large deposits of material in the interface. However, switching to a polymeric amino phase gave stable performance and baseline separations of fructose, glucose, and sucrose (Fig. 2). Now we could investigate the adulteration of honey by corn syrup using IRMS. With HPLC, we avoid adding large numbers of exogenous carbons from derivatization to generate the volatile species that would be required for GC–IRMS analyses. Table 1 illustrates data from this type of experiment. High fructose corn syrup has a markedly different natural abundance of ^{13}C, so that the isotope ratio of honey adulterated with even 25% corn syrup is altered considerably.

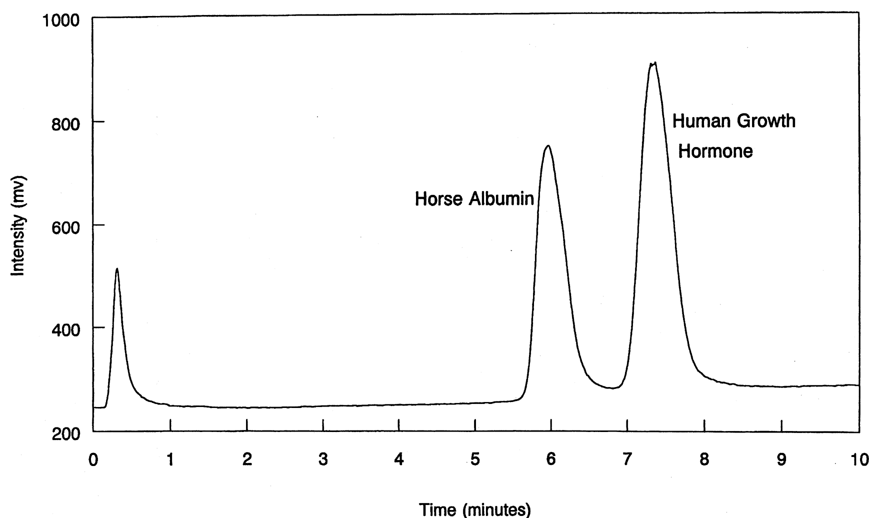

Fig. 1. Isotopic analysis of a sample of human growth hormone. The injection contained 100 pmol of growth hormone and 30 pmol of albumin The mobile phases used with the Poros R2 column were: A=0.1% trifluoroacetic acid (TFA) in water; B=0.1% TFA in acetonitrile. The gradient used was 30% B for 2 min followed by an increase to 70% in 10 min. The flow-rate was 1 ml/min. This tracing shows the m/z 44 channel that monitors $^{12}CO_2$.

Rarely has ion-exchange chromatography been interfaced with any type of MS system, but we have developed a scheme to examine isotope effects in the enzymatic hydrolysis of GTP. Fig. 3 shows the excellent separation of GTP, GDP, and GMP with an ion-exchange column and a gradient of 1.0 *M* NH_4OAc. Such a high concentration of this volatilizable ammonium salt appears to be completely removed by the interface so that the IRMS system did not have difficulty with this mobile phase.

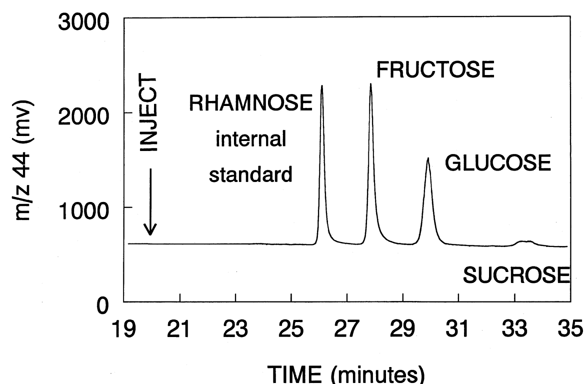

Fig. 2. Analysis of sugars in a 500 μg sample of honey. The Asahipak NH2P column was used with an isocratic 1 ml/min flow of a mobile phase containing water–acetonitrile (25:75). This chromatogram was selected from a series of chromatograms, so the time of this injection is 19.3 min as indicated by the arrow. The sucrose peak just after 33 min is too small to yield an accurate isotope ratio.

Finally, we present a new application of the combination of size-exclusion chromatography (SEC) and CRI-MS, a scheme we described recently [12,13]. Here, we examine the composition of a synthetic conjugate vaccine. In this construct, the polysaccharide component has been initially activated by a bifunctional linker, adipic acid dihydrazide, uniformly labeled with ^{13}C. Even adding six atoms of ^{13}C to a polysaccharide of $M_r >$ 10 000 was expected to elevate the isotope ratio markedly. If this was true, then after completing the conjugation to a protein moiety having natural ^{13}C

Table 1
^{13}C abundance from honey samples

Sample	$\delta^{13}C$ (‰)[a]	
	Fructose	Glucose
Honey	−2.93±0.41	−2.84±0.51
Honey+25% HFCS	−0.06±0.42	2.44±0.72

[a] These isotope ratios (means±SD) are the differences between the observed values and an internal isotopic standard of rhamnose having a $\delta^{13}C$ of −23.95‰. For each observation, $n=6$ or 7.

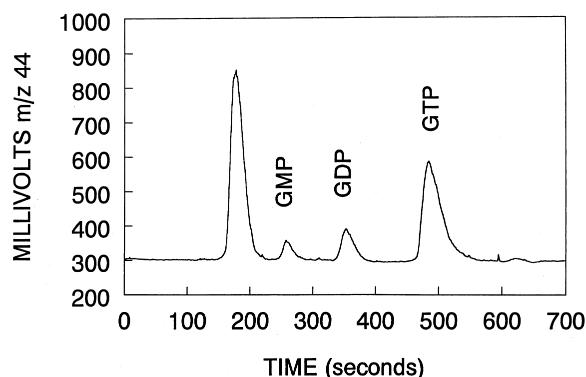

Fig. 3. Separation of a hydrolyzed sample of guanosine triphosphate using ion-exchange. The Bio-Rad ion-exchange column was used at 1 ml/min flow. Solvent A was water and solvent B was 1 *M* NH_4OAc. We used a 10 min gradient from 40% B to 80% B. The large carbon-containing (but not ^{13}C-enriched) peak seen before GMP is presumed to be sodium acetate, a non-volatile species formed from sodium in the sample and acetate in the mobile phase. We have not carried out a calibration for GTP, so the actual amount of the sample in this analysis is not known. By comparison with the CO_2 signals from other pure materials we believe this chromatogram represents 1–10 μg of GTP.

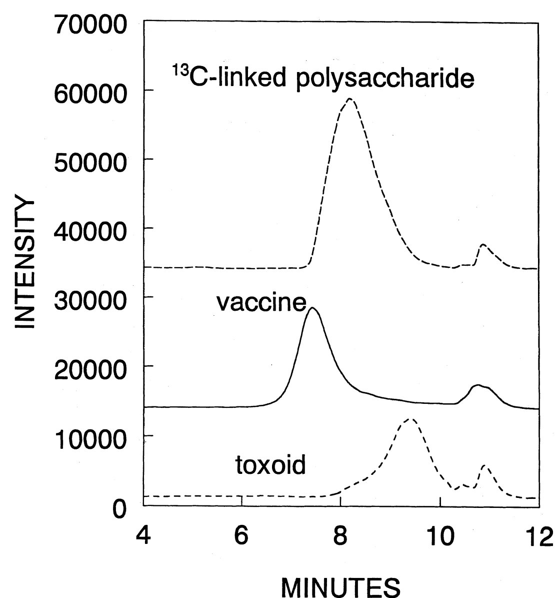

Fig. 4. Size-exclusion chromatography of a conjugate vaccine and its component parts. Because of the developmental nature of these materials, the concentrations of none of the components was determined. The GPC Linear column was operated isocratically at a flow-rate of 0.3 ml/min with a mobile phase of 100 m*M* NH_4OAc at pH 6.5. Each tracing contains some low-M_r carbon-containing species at 11 min. The presence of these impurities would affect the isotope ratio if the samples were run without some type of chromatographic separation. The baselines of the upper two tracings have been offset for clarity.

abundance, the resulting vaccine would have an isotope ratio that fell between the natural abundance of the protein and the elevated ^{13}C content of the polysaccharide. The isotope ratio of the vaccine would indicate the proportions of the protein and polysaccharide. Fig. 4 shows chromatograms of a vaccine produced from a *Clostridium welchii* protein toxoid and a polysaccharide derived from *Haemophilus influenzae* b (Hib) and its two component moieties analyzed by SEC–CRI–IRMS. Determining the composition requires knowledge of the molecular masses of each moiety, and this information is obtained from the SEC chromatograms using suitable M_r standards. The *Clostridium welchii* toxoid had a $M_r = 28\,300$ and the linked Hib polysaccharide had a M_r of 11 000. These M_r values along with the IRMS data allowed an estimation of composition of 38% Hib and 62% *Clostridium welchii* toxoid.

Finally, we continue to use HPLC–CRI–IRMS to develop a stable-isotope based method for measuring DNA synthesis rates [14]. The labeled precursor is incorporated into a cell's DNA, that DNA is extracted and hydrolyzed to nucleosides, and the nucleosides are analyzed with IRMS after reversed-phase separation. We have tested three labeled precursors to one or more components of DNA in HEP G2 human hepatoma cells in culture to evaluate the cellular and economic efficiencies of each labeled species. [^{13}C]Glycine, that selectively labels purines, is the most economical because of relatively low concentrations of glycine in plasma or growth medium and [^{13}C]glycine's relatively low cost. [^{13}C]Glucose, that becomes deoxyribose and labels all components of DNA, is efficient but not as economical as glycine. Despite the relatively low cost of ^{13}C-labeled material, glucose has 30-fold higher concentrations in medium or plasma, requiring 30-fold more material to obtain a similar degree of enrichment as with glycine. The third labeled precursor, [^{13}C]thymidine, generates high enrichment of thymidine from DNA, but is much more expensive per mg than glucose or glycine. However, thymidine exists at only micromolar concentrations

in plasma, allowing much lower amounts to be used. Per unit of enrichment, thymidine is somewhat less expensive than glucose. Combining this information regarding cellular efficiency with in vivo kinetic and bioavailability studies should guide us to an optimal scheme for measuring cellular replication rates in vivo.

4. Conclusions

We find that interfacing IRMS to HPLC greatly widens the range of applications for this very powerful instrument and removes the requirement of derivatization for non-volatile species. A variety of applications for isotope-ratio monitoring involving primarily GC introduction have been published [1,15]. The availability of precise isotopic measurements for macromolecular and non-volatile species should greatly simplify isotopic tracer experiments by lowering the amount of tracer needed, removing the requirement for derivatization, and, perhaps, even eliminating the need to hydrolyze biopolymers to obtain information about their enrichment. Exploring new combinations of HPLC methodologies with IRMS should further expand its uses.

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