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Element- and isotope-specific detection for high-performance liquid chromatography using chemical reaction interface mass spectrometry

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Abstract

We have developed a combination of high-performance liquid chromatography (HPLC) and the chemical reaction interface mass spectrometry (CRIMS) method by using a Vestec Universal Interface (UI). This interface provides the extremely high degree of solvent removal that the CRIMS process requires. In doing so, we have produced an HPLC detector with the ability to carry out element- and isotope-selective analyses with detection that is inherently: linear, structure-independent, sensitive, selective, comprehensive and flexible. The characteristics of the instrumentation and its performance are described.

Keywords: Detection, LC; Mass spectrometry; Chemical reaction interface mass spectrometry; Element-specific detection; Isotope-specific detection

1. Introduction

The chemical reaction interface for mass spectrometry (CRIMS) was first developed by Markey and Abramson in 1982 as a way of utilizing the mass spectrometer as an element (carbon) and an isotope (^{14}C) selective detector for gas chromatography [1]. Chace and Abramson further developed the technique to encompass a range of stable nuclides [2,3] and were the first to show the application of this technique to drug metabolism studies [2,4]. The basic principle of CRIMS is for the chromatographic effluent to flow into a microwave-powered reaction cell where the molecular species are broken down to elements. The continuous addition of a reactant gas to the microwave-induced plasma results in formation of simple gaseous reaction products that are characteristic of the elements contained in the original molecule. Conventional electron ionization (EI) mass spectrometry is then used to detect elements or isotopes in these reaction products. The chemistry within the microwave-induced plasma can be modified by using different reactant gases.

In many cases, the added gas is used to form oxidation products of the decomposed molecules of interest. The best oxidizing gas is SO_2 [2] that

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exhibits a wider dynamic range for carbon, a higher yield for the monitored oxidation product of nitrogen (i.e., NO), and extends electron filament lifetime compared to oxygen. When SO_2 is used, CO and CO_2 are produced from carbon-containing species and N_2 and NO are produced from nitrogen-containing species [2]. ^{13}C-Labeled compounds are detected at m/z 45 as the product $^{13}CO_2$ and, analogously, ^{15}N-labeled species are detected at m/z 31 as ^{15}NO. Selective element detection has been demonstrated for H, N, C, S, Br, Cl, Se and P, which give product ions that are unique and diagnostic for the atoms in the original molecule [4–10]. The ability to control the chemistry within the reaction cell, and the inherent capabilities of the mass spectrometer to separate ions based on their mass-to-charge ratios, are the bases of the selectivity of the CRIMS technique.

The major advantage of HPLC as an analytical separation technique lies in its acceptance of nonvolatile, polar, or thermally labile molecules without extensive sample work-up or derivatization procedures. The rapid growth of HPLC in recent years has largely been the result of advances in column technology. The importance of HPLC to biological analyses seems to have surpassed GC due to its applicability to polar nonvolatile compound analysis. In contrast to GC, however, the range of selective detectors for HPLC is limited. In particular, element- or group-selective detectors which are of great value in GC, such as the nitrogen–phosphorus, flame photometric sulfur, or electron-capture, have little current practicality for HPLC.

Like radioisotope detection, an important characteristic of the CRIMS technique is that in the process of decomposing molecules of interest and converting them into small gaseous molecules, one creates a selective detector which is independent of compound structure. This insures that detection is not only selective and universal, but also exhibits response factors which are independent of structural variations in the molecule. This is unlike other commonly used HPLC detectors which rely on physical properties of the intact molecule for their detection efficiency and are therefore influenced by structural differences in the molecule.

The interface between the separation method and the microwave-induced plasma reaction chamber is the primary limitation of coupling CRIMS to various separation methods. The ability to use gas-phase detectors with HPLC relies on the efficient removal of the solvent from the HPLC sample stream. We have previously described some applications and performance characteristics of this HPLC–UI–CRIMS system [11–14], but have not given details of its development and optimization strategies, nor described the full range of materials with which it can work. These points are contained in the present article. This type of selective detection capability coupled with high-performance separation methods will likely have a significant impact on a wide variety of chemical disciplines including biomedical and environmental research. To illustrate these possibilities, a number of representative molecules have been examined by this new instrument configuration.

2. Experimental methods

2.1. Materials

Unlabeled samples were obtained from Sigma (St. Louis, MO, USA). [4,5,6,8-$^{13}C_4$, 9-^{15}N]Deoxyguanosine and guanine were generous gifts from Dr. Suresh N. Yeola of Vanderbilt University (Nashville, TN, USA). The rest of the [^{15}N]L-glutamic acid and [2-^{13}C]cytosine and [1-^{13}C]glucose samples were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The HPLC solvents were purchased from EM Sciences (Gibbstown, NJ, USA), and had 0.1 ppm or less residue after evaporation. High-purity grade (99.995%) He gas was obtained from Air Products (Allentown, PA, USA) or Roberts Oxygen (Rockville, MD, USA). The SO_2 used was obtained from Matheson Gas Products (East Rutherford, NJ, USA). The one-hole alumina insulators (5 inches long, 1/4 inch O.D., 3/32 inch I.D.; 1 inch is 2.54 cm) in which the reactions take place are obtained from Scientific Instrument Services (Ringoes, NJ, USA) and are specified 0.250±0.002 inch O.D. to permit the use of Swagelok (Crawford Fitting, Crawford, OH, USA) fittings. The Vespel 10% graphite ferrules were obtained from Alltech (Deerfield, IL, USA) and the uncoated fused-silica transfer lines were purchased from J&W Scientific (Folsom, CA, USA). The C_{18}, 150×4.6 mm I.D., Kromasil column was obtained from Eka-Nobel

(Bohus, Sweden) and for protein separations, a Poros R1 10×2.1 mm column from PerSeptive Biosystems (Framingham, MA, USA) was used.

2.2. Description of the apparatus

The block diagram shown in Fig. 1 gives an overall view of the HPLC–CRIMS system. The key components are: the HPLC system, including pumps, injector, column and an optional UV detector; the particle-beam interface, incorporating the Universal Interface (UI) and momentum separator; the CRI, which requires the microwave power supply, the microwave cavity, the reactant gas supply and variable leak valve; and the mass spectrometer and its data system. The HPLC, the mass spectrometer, the UI, the microwave cavity and the data system require no modifications to perform element- and isotope-selective detection of HPLC effluents. The momentum separator has been modified as will be described below.

The HPLC unit consists of two SSI 222C pumps and a 232C gradient controller (SSI Instruments, State College, PA, USA) and a Rheodyne Model 7125 injector (Alltech). The 100 W, 2450 MHz microwave power supply was purchased from Opthos (Model MPG4, Rockville, MD, USA). The microwave cavity was manufactured by Vestec (Vestec Mass Spectrometry Products, Houston, TX, USA) [12].

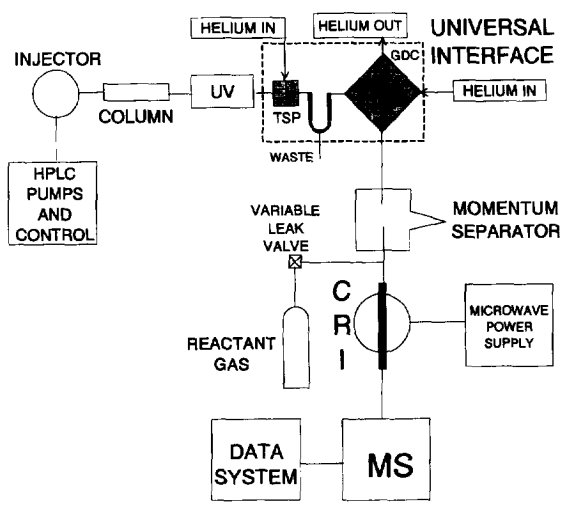

Fig. 1. Schematic of the HPLC–UI–CRIMS system.

The "Universal Interface" (UI), as it is called, was developed by Vestec and is based on the formation of a highly focused particle beam using thermospray (TS) vaporization followed by a two-stage desolvation process which efficiently removes the volatile solvent from the effluent stream [15]. The UI delivers a dry particle beam to the mass spectrometer with very little residual solvent. It operates at normal-bore HPLC flow-rates and uses a high He gas flow to carry the particle beam through the apparatus and to a mass spectrometer or other gas-phase detector. The two-stage desolvation process consists of a desolvation chamber and a longitudinal gas diffusion cell (GDC). The desolvation chamber removes the bulk of the solvent through condensation of the effluent on the walls of the spray chamber as it is partially vaporized using the TS vaporizer. Condensates are collected at the bottom of the condensing tube and are pumped away to waste using a peristaltic pump. The particle beam continues through the system under the influence of a high He flow which serves as a carrier as well as a focusing medium for the particle beam. Particles stay in the center of the bulk gas flow due to their very small longitudinal diffusion and are therefore highly defined. Further desolvation of the wet particle beam is accomplished using a heated, membrane-based, longitudinal gas diffusional cell.

Here, gaseous species having relatively high diffusibility properties encounter the walls of the apparatus where they pass through a porous membrane. These volatile species are then carried away to a vent by a countercurrent sweep gas flowing on the opposite side of the membrane. The dry particles then pass through a momentum separator which removes much of the remaining solvent vapor and a large portion of the He carrier gas. The particles are then directed to the desired gas-phase detector such as the mass spectrometer, flame ionization detector, or CRIMS system, depending on the type of sample information that is desired.

For the HPLC–CRIMS system, a single-stage momentum separator is used rather than the two-stage separator found in the standard HPLC–MS configuration. In the single-stage system, both the jet nozzle and the skimmer use a diameter of 0.6 mm. The helium and solvent removal is accomplished by pumping the body of the momentum separator with a mechanical pump and selectively sampling the par-

ticle-enriched center of the gas flow using the nozzle/skimmer configuration. The function of the skimmer is similar to that of a jet separator which is commonly used in packed column GC–MS. It acts as a particle-beam enrichment device and also as a restriction to bulk gas. The momentum separator is heated to 130°C to minimize condensation of the sample or residual solvent vapor in this region.

After exiting the momentum separator, the particle beam is mixed with SO_2 and then enters the alumina reaction tube where it is degraded to elemental form by the microwave-induced plasma. The alumina tube we use here has a larger I.D. than the 1/16 inch I.D. used for GC–CRIMS [16]. Although the particle beam is collimated by the 0.6-mm diameter skimmer of the momentum separator, it no longer has a high helium flow to focus it. The larger I.D. is used to maximize the chance that the particles enter the plasma without striking any surfaces. After exiting the high-energy zone, the elements react with the atoms from SO_2 to form small gaseous molecules.

When first setting up, tuning is accomplished by changing the volume of the microwave cavity using any of the movable components of the end plates [6] while minimizing the reflected energy as indicated on the microwave power supply. The cavity is tuned to deposit the maximum amount of energy in the cavity volume thus sustaining the plasma. In order to initiate the plasma, a spark from a Tesla coil or high-frequency generator (Model BD-40E, Electro-Technic Products, Chicago, IL, USA) was sometimes required. For that purpose, an additional 1/16 inch Swagelok connector was incorporated in the outflow end of the momentum separator mounting flange through which a thin wire in an insulating sleeve of Teflon extended near the He stream. The most efficient ignition was achieved with only He flowing into the CRIMS apparatus and the microwave power supply tuned for minimum reflected power.

The reaction products are then transmitted to the mass spectrometer through a 38-cm-long section of uncoated 0.53 mm I.D. fused-silica capillary tubing. The region between the alumina tube and the mass spectrometer is kept heated at 160°C to prevent condensation of elemental sulfur. A more detailed schematic of the momentum separator and its coupling to the CRI has been published elsewhere [12].

This research was done in two stages. The first involved the design engineering and a limited amount of applications, and the second phase focused on beta-testing what was a tentative interface configuration. In the development stage, the mass spectrometer used was a Vestec Model 201 quadrupole equipped with an EI ion source. The ion source temperature, electron energy and emission current were set at 200°C, 70 eV and 100 μA, respectively. Under particle-beam HPLC–CRIMS operation, pressure in the source region was $(2–4)\cdot 10^{-6}$ Torr (1 Torr=133.322 Pa). The data systems used for mass spectrometer control and acquisition were from Teknivent (Maryland Heights, MO, USA), either Vector 1 or Vector 2 Models. The mass spectrometer was operated in the selected ion monitoring (SIM) mode acquiring ion responses at m/z values of 30, 31, 44, 45 and 76. The identities of these ions correspond to ^{14}NO, ^{15}NO, $^{12}CO_2$, $^{13}CO_2$ and CS_2, respectively. The ions related to NO and CO_2 are monitored for isotope-selective detection of compounds containing nitrogen or carbon. We acquire m/z 76 to monitor the integrity of the plasma [2,16]. Flow injection analyses were carried out using a solvent system of 50:50 (v/v) water–acetonitrile. The data in Fig. 2, Fig. 3 and Fig. 7 were obtained with this apparatus.

The second half of these experiments used the same HPLC–UI system, but with an Extrel (Pittsburgh, PA, USA) C50-400 quadrupole mass spectrometer with ion source temperature, electron energy, and emission current set at 200°C, 70 eV and 3 mA, respectively, and a Teknivent Vector 2 data system. As previously described [16], this MS system can be configured with a higher-frequency RF power supply to increase resolution and sensitivity. Because of the high abundance of O_2 from the reactant gas, it is difficult to completely separate m/z 31 from m/z 32 without some overresolving. This is much more easily accomplished with the Extrel. The data in Figs. 4–6, as well as the limit-of-detection data were obtained with this instrument.

Equally critical is the adjustment of the helium flow-rates. The optimum flow-rates with HPLC–CRIMS may be different from the factory-recommended settings which were optimized for HPLC–MS analyte transmission. In HPLC–CRIMS, analyte transmission is certainly important, but an equally important parameter is the effective removal of the

solvent vapor. To optimize, we look at the m/z 44 channel and measure the ratio of the analyte peak height to the baseline intensity, the latter indicates residual concentration of the organic component of the HPLC solvent. This optimization may be done with flow injection or with a column, using a solvent that is about 50% organic and a flow-rate that is comparable to what will be used analytically. We find that to obtain the driest particle beam with good analyte transmission, the He flow that carries the particle beam is set to approx. 4 l/min and the countercurrent He flow is approximately 2.5 l/min.

2.3. Optimization of HPLC–CRIMS

2.3.1. UI

There are a number of parameters to be controlled when using the UI. These include the flow of carrier and countercurrent sweep gas, and various temperatures, such as those related to the TS vaporizer, the spray chamber, the countercurrent GDC and the momentum separator. The most critical parameter related to the performance of the UI is the TS vaporizer temperature. This was optimized by maximizing the response for direct injections of cytosine. The other UI parameters were adjusted similarly. All of the experimentally controlled parameters had relatively broad maxima so the system was set up quickly and reproducibly. The optimized conditions obtained using cytosine and a 50:50 water–methanol solvent were: TS control temperature=103°C; TS tip temperature=150°C; spray chamber temperature 1=48°C; spray chamber temperature 2=35°C; membrane temperature=52°C; momentum separator temperature=130°C.

2.3.2. CRIMS

CRIMS was optimized with regard to good chemistry and maximum signal which in most cases go together. Good chemistry is investigated by observing the enrichment-only chromatogram (EOC) which is generated when the Teknivent function CALC uses the equation: intensity at 45−intensity at 44·0.0119, where 0.0119 is the natural abundance of $^{13}CO_2+{}^{12}C^{17}O^{16}O$ [2]. Injections of unenriched compounds should give no signal in this channel. Causes for bad chemistry include: (a) insufficient reactant gas; (b) inadequate pressure in the reaction tube and (c) contamination of reactant gas either through leaks or in the cylinder itself. These are each explained below.

Inadequate reactant gas or analyte overload.

When there is insufficient gas for the concentration of the analyte, one gets partial combustion, pyrolysis, and/or reduction products. A lower than expected CO_2 signal will be seen. When SO_2 is the reactant gas, the signal at m/z 76 (CS_2) is the most sensitive indication of inadequate reactant gas. As a result, the presence of a peak at m/z 76 upon injection of a sample is undesirable and indicates the need for more reactant gas in the system. Furthermore, the m/z 45/44 ratio of the CS^+ fragment is higher than that for CO_2 and this will lead to a false positive result in the CALC-generated EOC. The amount of SO_2 should be increased until no CS_2 is formed when 1–10 μg injections of a test compound are injected. The color of the plasma is also diagnostic and should be examined from time to time. He alone should be peachy-yellow, but significant leaks of air give a light violet color due to the presence of nitrogen. The increasing concentration of SO_2 changes the color to a dull grey-yellow.

Low pressure in the reaction tube.

The pressure must be high enough to ensure sufficient collisions to cause the breakup of every particle in the beam and to break all bonds in the analytes. A restriction produced by using a fused-silica transfer line to the mass spectrometer increases outflow resistance on the MS side. The pumping of the momentum separator can also be reduced by using a choking valve on the mechanical pump. The third method of increasing pressure is increasing the reactant gas past the point where the color change has occurred, but this strategy quenches the plasma, thereby lowering the effective temperature and abolishing the basic CRIMS reaction chemistry. Insufficient pressure in the CRI will be noted by an incorrect 45/44 ratio even when there is no response in the m/z 76 channel. This is likely due to species such as $C_2H_5O^+$ at m/z 45 that represent incomplete combustion. At extremely low pressure one will see mass spectrometric evidence of intact analyte species much as if the microwave energy was not turned on.

Contamination of carrier or reactant gas.

The major contaminant is air. Increased nitrogen causes high levels of NO and thus measurement of nitrogen becomes difficult because the m/z 30 signal is high or off-scale. Sensitivity also severely suffers as nitrogen competes with the SO_2 for the analyte elements. Solutions to this problem include eliminating all leaks, prepumping the lines from the gas cylinder and regulator, and obtaining a specially prepared SO_2 tank [16] where the contamination of the head space has been eliminated by a substantial purge of the tank contents.

3. Results

The aims of this study were to develop a microwave-powered CRIMS interface for normal-bore HPLC, and to demonstrate the satisfactory performance of the system with a wide variety of samples and chromatographic conditions. Compounds of biological interest are analyzed to demonstrate the accomplishment of these HPLC–CRIMS system goals. The test materials included isotopically labeled and unlabeled compounds ranging from small molecules to macromolecules. The analyses of these compounds demonstrate element-selective detection, selective detection of isotopically labeled compounds and reaction interface performance, each of which appear comparable to those capabilities when using GC introduction. Although qualitative aspects of the HPLC–CRIMS system are highlighted below, results we have obtained and published elsewhere evaluated its quantitative potential [12–14].

The initial experiments to implement HPLC–CRIMS with the Universal Interface were very promising. While other commercially available particle-beam interfaces might successfully desolvate the droplets, they would not remove enough solvent vapor from the gas stream to allow for proper operation of the chemical reaction interface. For this reason, it is necessary that some sort of additional desolvation process be used to reduce solvent concentrations to a nearly vanishing level. Efficient removal of solvent, including water and organic modifiers, is accomplished using the UI and momentum separator. The background is very low. Thus, quenching of the plasma by solvent vapor is not occurring. The solvent-related increments in baseline are small when compared to the sample signal. With the moving belt device we had previously crafted, the amount of solvent vapor entering the CRI was also low, but its overall potential as a routine device was unsatisfactory [17].

Injections of both labeled and unlabeled samples were made in order to show element-selective detection of carbon and isotope-selective detection of ^{13}C. The top panel in Fig. 2 represents a 'universal' response for carbon at m/z 44, corresponding to the formation of $^{12}CO_2$ in the reaction interface. This is analogous to the response of a flame ionization detector in GC that offers universal detection for carbon-containing compounds. Even the labeled compounds, which were synthesized having one ^{13}C atom per molecule, are mostly made up of ^{12}C so they also give a large response at m/z 44. The relative areas for each peak in the m/z 44 chromatogram are proportional to the amounts of ^{12}C injected. Panel B shows the selected ion chromatogram of m/z 45. This response follows the formation of $^{13}CO_2$ in the reaction interface. The unlabeled samples exhibit peaks at m/z 45 due to naturally occurring ^{13}C. The isotopically labeled compounds clearly give a much

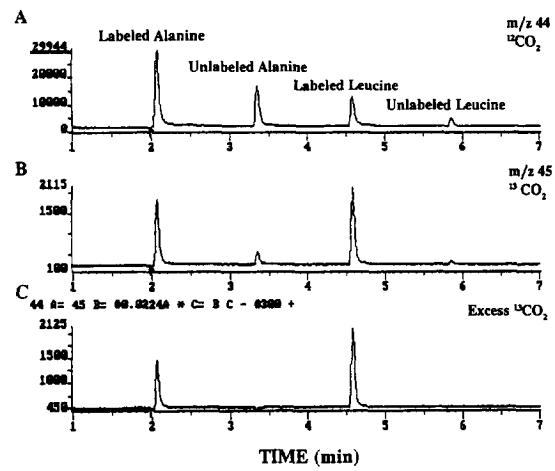

Fig. 2. Flow injection analyses of unlabeled and ^{13}C-labeled amino acids with the Vestec mass spectrometer. Panel C shows the $^{13}CO_2$ response corrected for naturally occurring ^{13}C and ^{17}O. The amounts of material injected are: labeled alanine, 10 μg; unlabeled alanine, 5.6 μg; labeled leucine, 4.2 μg; and unlabeled leucine, 1.1 μg.

higher $^{13}CO_2$ signal due to their enrichment. To more effectively demonstrate the isotope-selective response of the labeled species, the signal of naturally occurring ^{13}C and ^{17}O can be subtracted from the m/z 45 response. This subtracted value can then be plotted as the 'enriched' m/z 45 response by using the CALC function [2].

Panel C represents the response for m/z 45 corrected for the contribution of naturally occurring ^{13}C and ^{17}O. The experimentally derived equation, which appears in the upper left corner of this figure, has a subtraction factor of 0.0224 as compared to a theoretical value of 0.0119. We believe the reason for this high factor is that something other than the isotopes of CO_2 are contributing to the m/z 45 signal due to an insufficient number of collisions in the reaction zone. Various improvements in system design enabled us to overcome this problem and subsequent results were obtained using the theoretically exact values.

Fig. 3 shows selected ion chromatograms for flow injections of unlabeled, ^{15}N-labeled, and a mixture of labeled and unlabeled alanine or leucine. Panel A represents the general response of $^{12}CO_2$ at m/z 44. Panel B represents the signal from ^{15}NO with the contributions due to naturally occurring ^{15}N and ^{17}O subtracted out. The m/z 30 channel, which is not shown, has a high baseline due to the high amount of

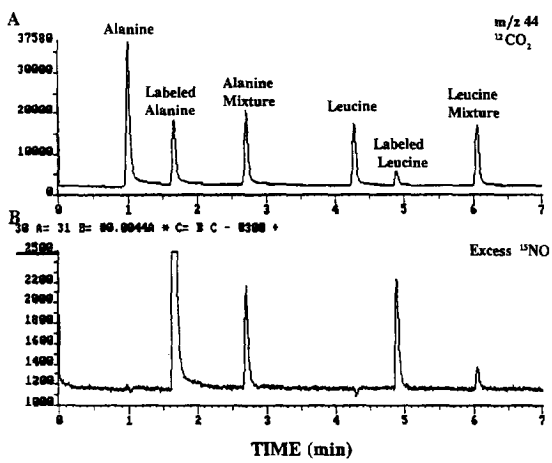

Fig. 3. Flow injection analyses of low (1–10 μg) amounts of unlabeled and ^{15}N-labeled amino acids using the Vestec mass spectrometer. Panel B shows the ^{15}NO response corrected for naturally occurring ^{15}N and ^{17}O.

N_2 in the system. Despite this, the ^{15}NO signal due to the injection of isotopically enriched samples is readily detected. Panel B simply shows enrichment-only detection for ^{15}N with peaks appearing only for the ^{15}N-labeled sample injections. The unlabeled sample gives no excess ^{15}NO signal. The mixture gives a smaller relative response than the labeled alanine and leucine injections because the labeled component makes up only a fraction of the total material injected. For both ^{13}C and ^{15}N detection, the peak areas were proportional to the amount of material in each injection from low nanogram to low microgram quantities.

Fig. 4 shows HPLC–CRIMS separation of a

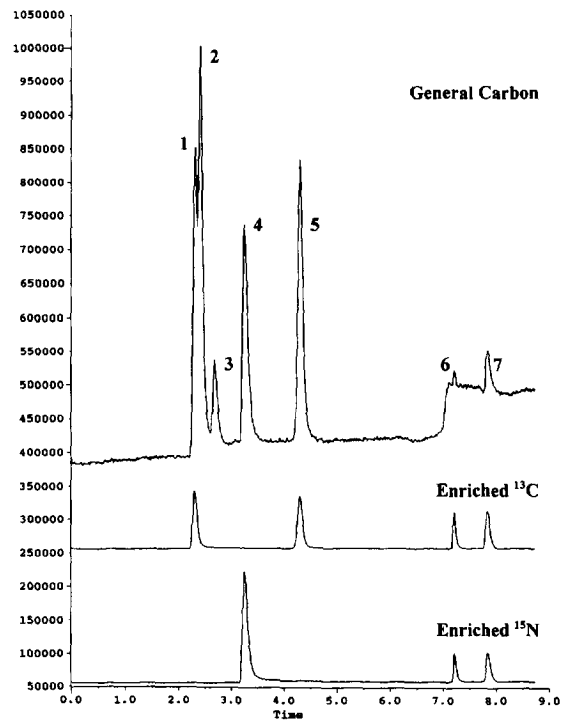

Fig. 4. HPLC–CRIMS analysis of small biomolecules with the Extrel mass spectrometer. In order of increasing retention times the compounds are: (1) 400 ng [^{13}C]glucose ($C_6H_{12}O_6$); (4) 300 ng [^{15}N]glutamic acid ($C_5H_9NO_4$); (5) 220 ng [^{13}C]cytosine; (6) 100 ng [$^{13}C_4$,^{15}N]guanine; (7) 50 ng [$^{13}C_4$,^{15}N]deoxyguanosine. A gradient of 1–100% methanol over 20 min at a flow-rate of 0.6 ml/min was used to separate the compounds on a Kromasil C_{18} column. For the sake of clarity, the baselines for the top-two traces have been moved. For the general carbon channel measuring $^{12}CO_2$, the baseline is actually 200 000 and the enriched ^{13}C baseline should be 60 000. Peaks 2 and 3 in the general carbon trace represent unlabeled contaminants.

mixture of small molecules. The general view shows narrow, symmetrical, peaks implying very little band broadening. An experiment comparing on-line UV with the MS profiles shows that there is minimal (less than one second) increase in peak broadening introduced by UI–CRI interface. This test mixture contained two ^{13}C-labeled compounds ([^{13}C]glucose, 1; [^{13}C]cytosine, 5), one ^{15}N-labeled compound ([^{15}N]glutamic acid, 4), and two multiple-labeled compounds ([$^{13}C_4$,^{15}N]guanine, 6; [$^{13}C_4$,^{15}N]-deoxyguanosine, 7). The top trace shows the general (unenriched) carbon channel and all the compounds are detected as $^{12}CO_2$. The middle trace shows the enriched ^{13}C-channel where compounds with that label are selectively detected (glucose, cytosine, guanine, and deoxyguanosine). Similarly, the bottom panel shows the enrichment-only ^{15}N channel and the three compounds with ^{15}N-labels are selectively observed (glutamic acid, guanine and deoxyguanosine). The selective channels were created with the theoretically-correct subtraction factors of 0.0119 for CO_2 and 0.00402 for NO. Peaks 2 and 3 are unlabeled contaminants as indicated by their having signals only in the general carbon channel. When we start with mobile phases containing less than 5% organic, a rise in baseline at about 7 min may be seen, but that too disappears when the enrichment-only traces are generated. The signals in the enrichment-only channels relate to the molar concentrations of the label. More involved quantitative HPLC–CRIMS studies published elsewhere have shown proportionality of signal to molar concentration [12,14]. The compounds analyzed here are small and highly water-soluble. HPLC–CRIMS can easily analyze such common, polar biochemicals.

Those molecules with very low absorbance, rendering UV detection inadequate, have been challenges for HPLC. We have analyzed a 500 ng sample of unlabeled erythromycin as an example of this group (Fig. 5). The very good signal/noise in the general carbon channel, ca. 1700, implies low ng detection limits. The contrast between the large peak in the carbon channel and the small peak in the general nitrogen channel (^{14}NO) is expected because the compound has 31 carbon atoms and one nitrogen. In CRIMS, the molar response factors for different elements are not identical, so the exact ratio of areas could only be interpreted by calibrating with a model

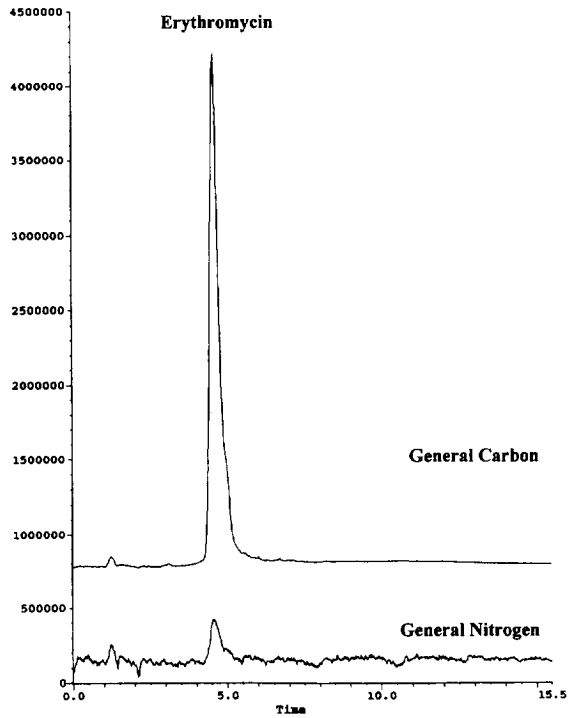

Fig. 5. HPLC–CRIMS analysis of erythromycin ($C_{37}H_{63}NO_{13}$, M_r 734, 500 ng injected) with the Extrel mass spectrometer. A solvent system of 100 m*M* ammonium acetate (A) and acetonitrile (B) and a gradient of 30–95% B over 10 min at a flow-rate of 1 ml/min was used to chromatograph the sample on a Kromasil C_{18} column. For clarity, the baseline for nitrogen detection was lowered. It should be 1 750 000.

compound. Using GC–CRIMS, this type of microchemical determination was done for C and Cl [8] and for ^{15}N and Cl [18].

Macromolecules are another group of compounds of interest. We find that the UI transmits molecules without an upper limit to their size. Fig. 6 shows the chromatographic separation with general carbon ($^{12}CO_2$) and nitrogen (^{14}NO) detection of three unlabeled proteins: insulin, cytochrome *c* and myoglobin. The amounts used here are 175 pmol insulin, 77 pmol cytochrome *c* and 60 pmol myoglobin. All three proteins are detected in both the nitrogen and carbon channels. Other macromolecules with a molecular mass beyond 700 000 have been transmitted and detected. With increasing interest in HPLC of macromolecules, the analytical potential for an HPLC detector with uniform carbon or nitrogen detection seems particularly high.

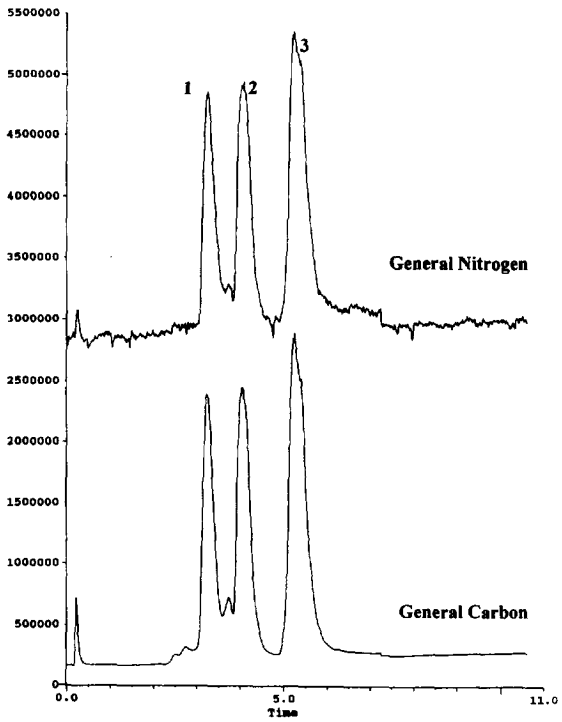

Fig. 6. HPLC–CRIMS analysis of proteins with the Extrel mass spectrometer. (1) 1 μg insulin (M_r 5700); (2) 1 μg cytochrome *c* (M_r 13 000); (3) 1 μg myoglobin (M_r=16 900). A solvent system of 5% acetonitrile–0.1% TFA in water (A) and 5% water–0.1% TFA in acetonitrile (B), with a gradient of 12–72% B in 10 min at a flow-rate of 1 ml/min was used to separate the proteins on a Poros R1 column.

The detection limit of CRIMS depends on the channels that are monitored and the number of labeled carbons or nitrogens the compound possesses. In a simple experiment we have determined the detection limit for glutamic acid with one of its nitrogens labeled. Using a S/N=3 definition, we could determine 0.8 ng of the intact amino acid. This is equivalent to about 5 pmol of nitrogen. For a ^{13}C label, the limit is 9 ng for ^{13}C-labeled cytosine or about 20 pmol. With this cytosine sample, the signal for $^{12}CO_2$ at m/z 44 is about 20-times the background noise, again suggesting a routine detection limit of low nanograms for most unenriched organic compounds. The lower detection limit for ^{15}N than for ^{13}C has been observed before [2] and is attributed to the lower baseline contributions of ^{15}N than of ^{13}C. This sensitivity advantage for labeled nitrogen over labeled carbon is in distinction to the detection of general (unlabeled) carbon and nitrogen, where the higher baseline at m/z 30 compared to m/z 44 restricts detection of nitrogenous materials compared to carbonaceous materials (see Fig. 5)

In Fig. 7, we plot the observed $^{13}C/^{12}C$ ratio vs. composition for various fractions of ^{13}C-labeled caffeine in unlabeled caffeine with the total amount of material injected being held constant at 4 μg. The fraction of labeled caffeine in the sample ranged from 0.0078 to 1 which corresponds with 31 ng to 4 μg of labeled caffeine injected. The m/z 45 response

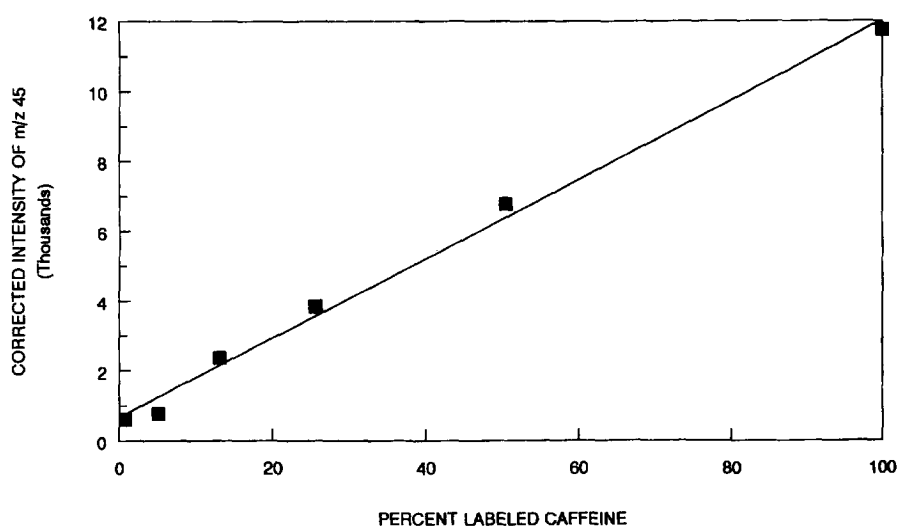

Fig. 7. Isotopic dilution of caffeine with the Vestec mass spectrometer.

is corrected for naturally occurring ^{13}C. The results obtained show a linear response for isotopic detection of ^{13}C.

4. Discussion

The research presented above gives details of the successful interfacing of the CRIMS apparatus with the Universal Interface and an EI mass spectrometer. These data show efficient solvent removal using the UI with specific detection of C- and N-containing compounds independent of chemical structure, and isotope-selective detection of ^{13}C and ^{15}N for a variety of polar nonvolatile species. Results obtained for known mixtures of labeled and unlabeled caffeine show linearity of response and demonstrate the quantitative potential of the technique. The CRIMS interface also showed no degradation of chromatographic resolution (i.e., no appreciable contribution to chromatographic band broadening). We have evaluated the Universal Interface down to 0.5 ml/min and have not seen any significant deterioration in signal intensity.

One potential application area of HPLC–CRIMS showing great promise is the determination of drug metabolites in biological fluids. The use of stable isotopes in this application is highly desirable because it eliminates the stringent guidelines required for the dosage of a radioactive drug to humans. In such studies, the parent drug would be labeled with a stable isotope. After the drug is administered and subsequently metabolized, the stable isotope is used as a "tag" that is common to the parent drug and metabolites. Specific detection of the isotopically enriched components thus identifies compounds as products of the labeled parent drug. Although mass spectrometry alone can be used to identify isotopically labeled compounds by a shift in the mass of the molecular ion or characteristic fragment ion, the variability and non-linearity of response observed when comparing various HPLC–MS particle-beam interfaces [19] implies that such quantitative studies will be relatively difficult.

CRIMS improves the utility of stable isotopes as tracers because it provides the same compound-independent detection that is normally only attributed to radioactive isotope detection. With HPLC–CRIMS, we have demonstrated these capabilities for metabolism studies of ^{13}C- and ^{15}N-labeled caffeine [11], [$^{2}H_3$]cortisol [12,13] and [$^{2}H_4$]acetaminophen [14]. These results show a broad range of metabolites detected, in particular intact conjugates, and at least three orders of magnitude dynamic range with metabolites at the 0.1% level being quantified [14]. HPLC–CRIMS is also capable of making isotope-ratio measurements on intact proteins at the femtomole level [20]. With these capabilities, HPLC–CRIMS could reduce the dependency on radiotracer methods both in preclinical studies as well as in human trials.

Previously reported research involving GC–CRIMS experiments show routine sensitivities in the low-nanogram to high-picogram range for each compound analyzed [2–10]. Based on these observations, HPLC–CRIMS should achieve routine sensitivity of at least tens of nanograms with the difference between GC–CRIMS and HPLC–CRIMS sensitivity arising primarily from the broader peaks and, secondarily, from the higher background in the HPLC–CRIMS technique. Other common problems with particle-beam HPLC–MS, such as low transmission to the ion source, decomposition upon vaporization, etc., are not problems in HPLC–CRIMS because the analyte molecules are completely decomposed and reformulated into simple gaseous species before being transmitted to the mass spectrometer. Our previous work with HPLC–CRIMS [12,14] and the data presented here confirm that the limits of detection are indeed in the low ng range. As one uses increasingly higher resolution chromatography, the detection limits should be proportionally reduced.

Another advantage of the HPLC–CRIMS approach is the freedom from derivatization. While many elegant GC–MS methods have been developed for isotopic analyses involving difficult analyses, the derivatization process may be burdensome, as well as counterproductive. For example, analyzing glucose as the acetyl-bis-butylboronyl derivative [21], urea as its bis-trimethylsilyl derivative [22], or glutamine as its tris- or tetrakis-*tert.*-butyldimethylsilyl derivatives [23] all add more carbons than the initial structures themselves contain. With HPLC introduction, these problems can be eliminated.

We have not yet implemented the element-selec-

tive capabilities of GC–CRIMS [4–10] into our HPLC–CRIMS work. There are no fundamental problems involved with these methods that would pose particular problems when attempted in the HPLC–CRIMS mode of operation. Work will be proceeding soon to generate element-selective HPLC detection schemes.

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