Application Note

Flow Scintillation Analyzer FSA-005

Metabolism Studies with On-line HPLC and Mass Spectrometry (MS) Interfaced with the Flow Scintillation Analyzer (FSA)

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Introduction

In the biological sciences, mass spectrometry is one of the most popular methods employed for the determination of the molecular weights and structures of metabolites. The popularity of mass spectrometry is due to the high sensitivity of the analytical method and the possibility of obtaining molecular weights and structure of metabolites directly from high performance liquid chromatography (HPLC) effluents without further sample treatment. The traditional and more time-consuming methods of structure analysis, including chromatography of metabolites, followed by chromatogram fraction collection and purification prior to submission of isolated metabolites to mass spectrometry, have been applied for many years. Advanced techniques include the on-line mass spectral structural analysis of metabolites directly off the HPLC column after detection of peaks of interest. This application note describes the state-of-the-art of on-line mass spectrometry of radioisotope-labeled metabolites following HPLC separation and FSA (Flow Scintillation Analysis) detection of chromatogram peaks of interest, also referred to as the hyphenated HPLC-FSA-MS analysis. This paper will provide examples from the recent scientific literature that demonstrate the advantages of on-line radioactivity and mass spectral molecular structure analysis of radioisotope-labeled metabolites purified by HPLC obviating the time-consuming step of compound collection from HPLC fractions.

HPLC-FSA-MS Instrumentation and Interfacing

The interfacing of liquid chromatography with mass spectrometry (LC/MS) is quickly becoming a preferred tool of chemists in the pharmaceutical and biotechnology industries. The combination of HPLC and MS permits the analysis of samples that have traditionally been very difficult, namely polar and/or thermally labile molecules. The addition of microvolume FSA to LC/MS constitutes a powerful tool capable of providing on-line precise and accurate quantitative and structural data for the molecular constituents of interest.

When the metabolism of a radioisotope-labeled compound is studied and the metabolites are separated by HPLC, flow scintillation analysis provides for the quantitative analysis of metabolites in terms of percentage of total recovered radioactivity. For example, when a parent compound labeled with a radioisotope, such as ^{3}H, ^{14}C, ^{33}P, ^{32}P, etc., is administered with a known radioactivity to a test animal or medium and the metabolites separated by HPLC, the percentage of the total radioactivity administered is automatically measured by the FSA prior to mass spectrometry. This is illustrated in this application note with the citation of recent research reports. Consequently, the use of FSA prior to mass spectrometry provides advantages over the UV detector, which include (i) irrefutable evidence that a certain HPLC peak is one of interest, (ii) the measurement of radioactivity from the isotope label is performed by the FSA without a miss, unless the isotope label is near or essentially at background levels, (iii) the FSA reports the radioactivity of the HPLC-separated parent compound and metabolite fractions in quantitative units of disintegrations per minute (DPM) providing valuable data for the quantitative percentages of total radioactivity administered to a test organism, and (iv) the FSA can store quantitative data on metabolites over a series of HPLC runs carried out over a time span to determine the time course of a metabolism study.

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Radioisotope tracers are commonly used in metabolic studies, and there remains the need to quantify the isotope label on the metabolites eluted from the HPLC prior to their molecular structure analysis via the mass spectrometer. FSA is the tool for this. The FSA provides the real-time radioactivity levels of metabolites as these are eluted from the HPLC column, and the radioactivity peaks from the FSA can provide the signal to initiate mass spectrometric analysis. The FSA is connected between the UV and MS if using a heterogeneous (solid) flow cell as illustrated in Figure 1. If a homogeneous (liquid) flow cell is used, the flow is split to both the FSA and MS. The heterogeneous flow cell uses a solid scintillant detector of radioisotope label (e.g. ^{3}H, ^{14}C) providing full recoveries of the HPLC eluate for subsequent MS analysis.

A popular heterogeneous flow cell for the FSA utilizes SolarScint™ (trademark of Packard Instrument Company, Meriden, CT), which is a solid scintillator that undergoes minimal compound binding in most cases, providing optimal peak resolutions, high detection efficiencies for ^{14}C (70%), and full sample recovery for mass spectrometry (*i.e.*, no effluent splitting). The homogeneous flow cell arrangement requires HPLC effluent splitting, because scintillation cocktail is mixed with effluent for radioisotope analysis. Stream splitting is often set to provide most of the stream to the FSA and a small portion to the mass spectrometer, because of the high sensitivity of mass spectrometers (pg/µL) that utilize electro spray ionization techniques for sample introduction. For example, Ramu et al. (2000) split the HPLC effluent at 1 mL/min in the ratio of one to nine to pro-

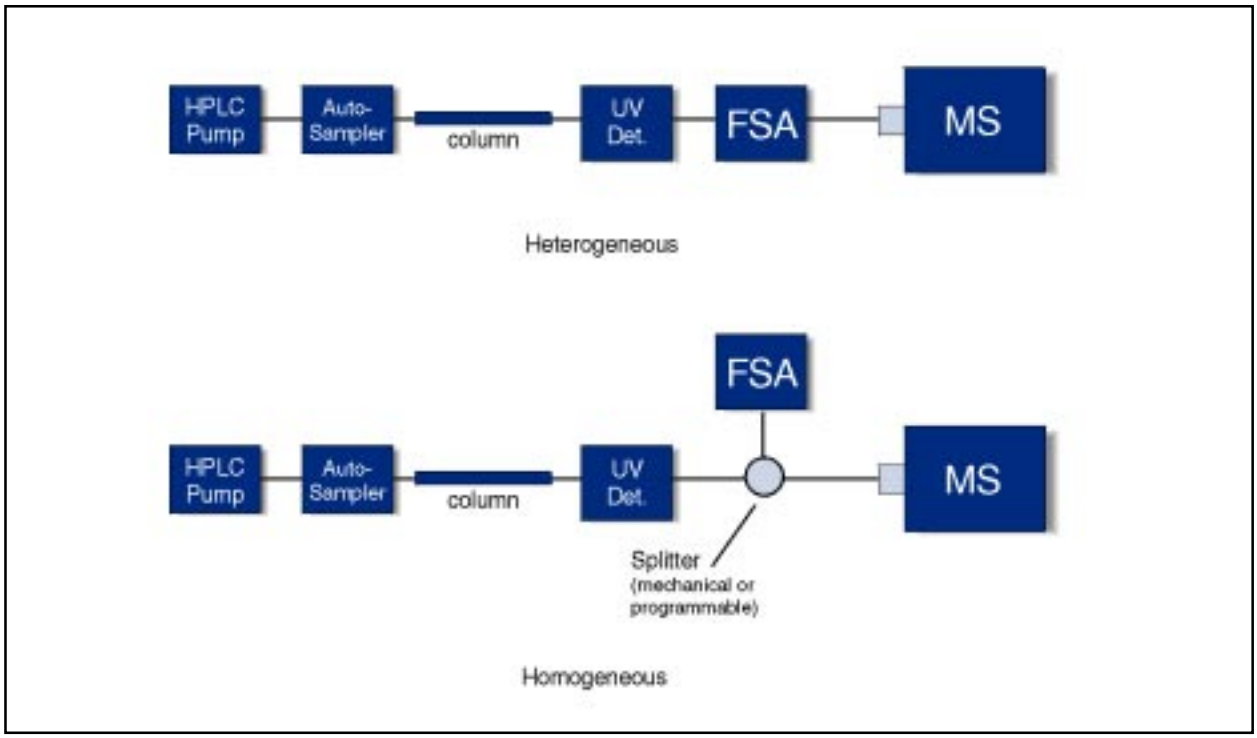

Figure 1.

Instrumental setup for the use of a Packard Flow Scintillation Analyzer (FSA) with on-line HPLC-MS. A heterogeneous flow cell in the FSA (upper) allows for the entire effluent from the HPLC to continue on to the mass spectrometer, whereas a homogeneous flow cell (lower) would require stream splitting prior to mass spectrometry. The homogeneous FSA setup is most often employed with on-line HPLC-MS, because only a fraction of the HPLC effluent is required for mass spectrometry, and higher detection efficiencies for radionuclide tracers are obtained with the homogeneous cells.

vide ca. 100 µL/min into the mass spectrometer and ca. 900 µL/min into the Packard Radiomatic 150TR FSA. Similarly Andersson et al. (1998) used a stream splitter that diverted ca. 80% of the HPLC effluent to a UV monitor and Packard Radiomatic A525 FSA and ca 20% of the effluent to the mass spectrometer. The homogeneous flow cell setup provides higher detection efficiencies (up to 45% for ^{3}H and 88% for ^{14}C) depending on the quench level of HPLC solvents.

Interfaces with the HPLC effluent and mass spectrometer must liberate the biochemical or bioorganic molecular species of interest (e.g. metabolite) from the aqueous solvent molecules and ionize the molecular species prior to mass spectrometric separation of the molecular ions and molecular ion fragments. This is performed most commonly by spray ionization (SI) techniques, which involve a combination of processes including spraying the HPLC effluent from a fine capillary into minute droplets, pneumatic heating with a drying gas, applied electric potential, and in some cases chemical ionization. The most common MS interfaces used in conjunction with HPLC are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). In the ESI method a nebulizer gas and electric field is introduced at the interface to produce charged droplets of the HPLC effluent. The combination of electric field energy and pneumatic heating via a warm concurrent dry gas stream cause the charged droplets of the HPLC effluent to subdivide and yield eventually single ionized molecules. APCI is a chemical ionization technique, which employs a mechanism similar to ESI. The differences exist in the establishment of a plasma of the nebulized HPLC effluent by a DC discharge at atmospheric pressure. Reactions between ions and molecules occur in the plasma to produce molecular ion species of the biochemical or bioorganic compounds present, which are then introduced via vacuum into the mass spectrometer. Spray ionization techniques yield high ionization efficiencies and consequently mass spectral detection limits as low as the pg/µL level. Also, spray ionization techniques are soft, which yield relatively minor molecular ion fragmentation providing accurate molecular weight determinations in the mass range up to 10^5-10^6 daltons, the sensitivity depending on the specific type of mass spectrometer used. The theory and principles of electrospray ionization are treated in detail by Kebarle and Tang (1993).

The molecular ions and molecular ion fragments produced via the spray ionization interface are separated in the mass spectrometer according to their mass-to-charge ratios (m/z) using electric and/or magnetic fields. Several types of mass spectrometers are available for use on-line with HPLC including time of flight analyzers, quadrupole ion filters, and quadrupole ion trap instruments. The characteristics of these instruments are described in detail by Lambert et al. (1998). A popular mass spectrometer used on-line with HPLC in metabolism studies is the triple quadrupole mass spectrometer illustrated in Figure 2, which is a tandem mass spectrometer (Andersson, et al., (1998), Boulton et al. (1999), Maggs et al., (2000), Riska et al. 1999, Noort et al. (1999), and Wormhoudt, et al. (1998). Tandem mass spectrometry is often abbreviated as MS/MS, because it consists of dual mass analyzers coupled in a tandem instrument useful in selectively separating the parent molecular ions from the product ion fragments. Dual mass analysis with the tandem analyzer is accomplished by first selecting the parent molecular ions after initial ionization with a sector magnet, and the molecular ions are further dissociated via collision with a gas such as He, Ne, N_2 or Ar referred to as collision induced dissociation (CID). The ion fragmentation products of CID are analyzed subsequently in the second mass analyzer according to their m/z and abundance. The tandem MS is highly sensitive and operates up to mass-to-charge ratios of 4000 described in detail by de Hoffman (1996).

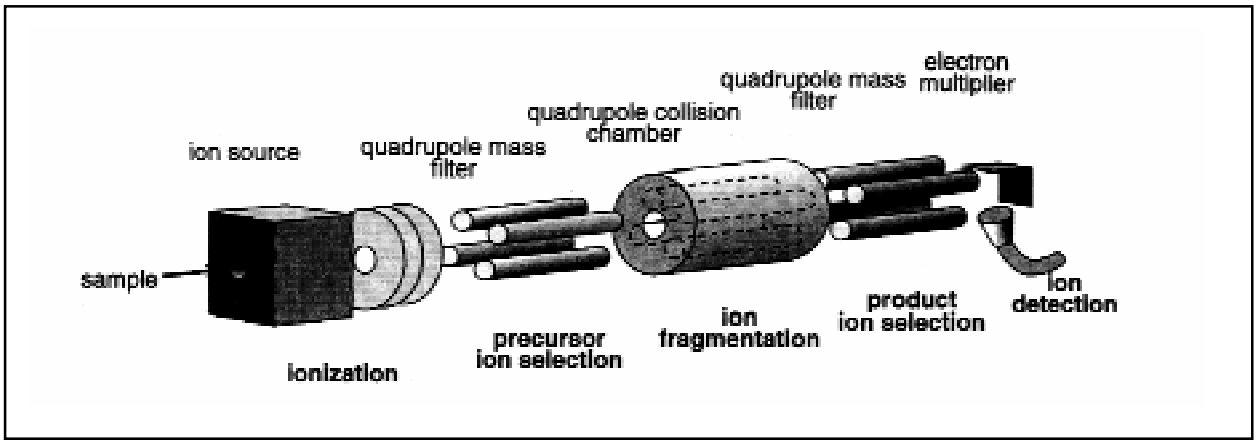

Figure 2.
Schematic diagram of a triple quadrupole mass spectrometer (From de Hoffmann, 1996).

HPLC-FSA-MS Representative Data

During the past two years, numerous research papers employing on-line HPLC-FSA-MS have appeared in the scientific journals. Only a few will be cited here and some examples of representative data described in this paper (Adas et al., (1998), Andersson, et al., (1998), Boulton et al. (1999), Kumar et al., (1999), Maggs et al., (2000), Noort et al. (1999), Ramu et al. (2000), Riska et al. 1999, Scarfe et al. (2000), Wynalda et al. (2000).

An interesting example can be taken from the work of Maggs et al. (2000), who studied the rat biliary metabolites of β-artemether (AM), an antimalarial endoperoxide. They administered [^{14}C]AM i.v. to rats (10µCi/kg) and collected bile hourly up to five hours. They submitted the bile to HPLC-FSA-MS including tandem HPLC-FSA-MS/MS. The HPLC eluate at 0.9 mL/min was split between the FSA and the LC-MS interface by taking only ca. 40 µL/min to the mass spectrometer. The LC eluate directed to the FSA was mixed with Packard Ultima-Flo™ AP scintillation cocktail (1 mL/min.). Electrospray ionization mass spectra were acquired with a tandem quadrupole mass spectrometer. The presence of ammonium acetate in the LC buffer produced the MS molecular ion (M) cationized with ammonium ion, i.e. $(M + NH_4)^+$. In tandem MS/MS, the collision-induced dissociation (CID) of the ammonium adducts of major metabolites was achieved with argon gas at a collision energy of 20 eV.

HPLC-FSA-MS of the [^{14}C]AM and bile metabolites provided quantitative analysis of the metabolites as well as evidence for structural confirmations. The HPLC radiochromatogram and radiometric quantification data of the HPLC peaks provided by the FSA are illustrated in Figure 3. The quantitative analytical data of metabolites, provided by the FSA, is a major advantage of FSA over UV detectors for the identification of HPLC peaks of interest.

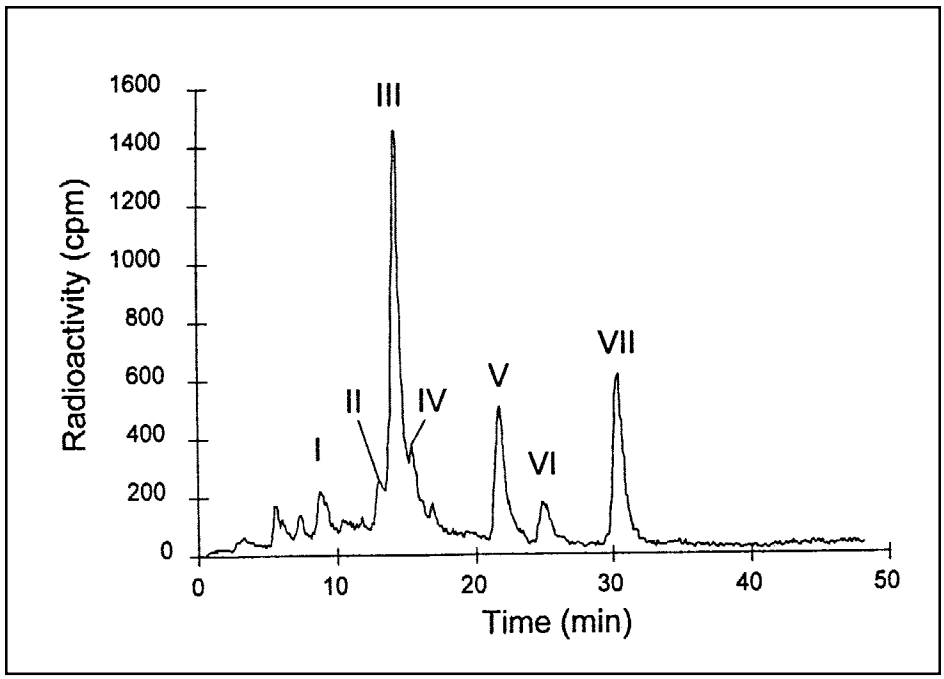

Figure 3.
HPLC radiochromatogram of the biliary metabolites (pooled 0- to 3-h collections) of [^{14}C]AM (35 µmol/kg, i.v.) in male rats. (From Maggs et al., 2000). Reprinted with permission of The American Society for Pharmacology and Experimental Therapeutics.

The proposed identities of the metabolites quantified in the above radiochromatogram were derived from data provided by the electrospray mass spectra of [^{14}C]AM metabolites and daughter ion spectra of the metabolites created by CID with tandem LC-MS/MS. An example of the daughter spectra of one of the major metabolites is illustrated in Figure 4. The daughter ions at m/z 476, 459, 265, 251, 237 and 219 were in agreement with the fragmentation pathway of compound **V**, a hydroxyAMglucuronide.

Future Trends
HPLC-UV-FSA-NMR-MS

For on-line spectroscopic analysis of natural products, Bailey et al., (2000), Hansen et al., (1999) and Shockcor et al., (1996) split the HPLC effluent in the proportions of 95% to the NMR spectrometer and the remaining 5% of the effluent to the mass spectrometer in light of the relative sensitivities of the two spectrometers. The NMR spectra were obtained using the stop-flow method with resonance signal acquisitions varying from several minutes to hours with a 500.13 MHz Bruker DRX-500 NMR spectrometer. The various acquisition times were dependent on compound concentrations off the HPLC column. Smith et al., (1999) also report the use of a splitter of HPLC effluent to the MS and NMR spectrometers. In the near future, we can expect to see a growing number of scientific reports with the hyphenated analytical methods of HPLC-UV-FSA-NMR-MS, as illustrated in Figure 5, for the on-line separation, radioisotope label analysis, and molecular structural elucidation of complex mixtures. As reported by Hansen et al., (1999) these techniques will cut the time needed to carry out such complex studies to short durations from one day to a few weeks compared to the span of months required when traditional techniques of compound isolation, purification, and subsequent spectroscopy are undertaken.

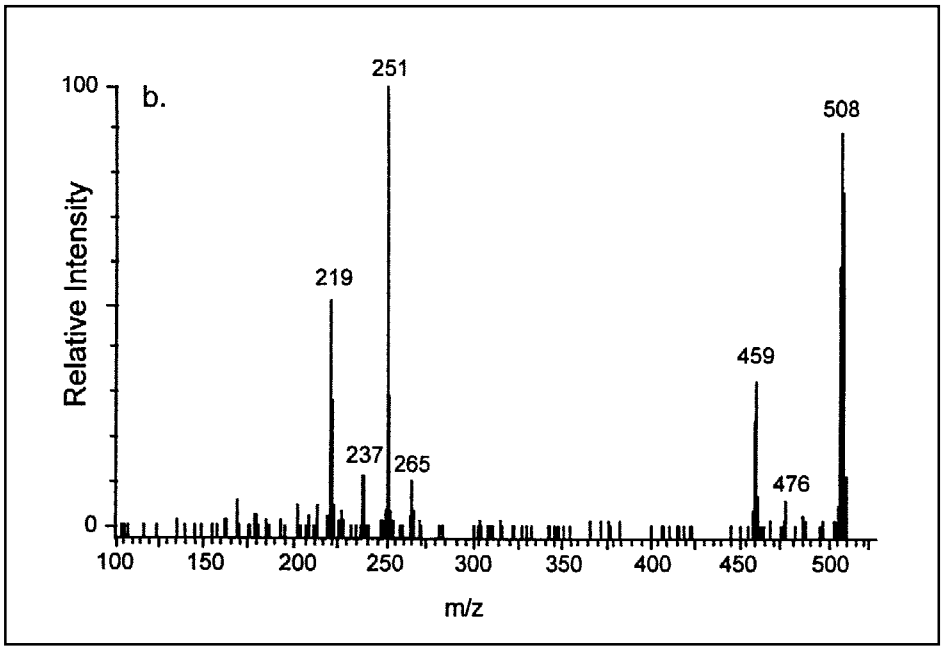

Figure 4.
Daughter spectra obtained by CID of $[M + NH_4]^+$ for compound V (hydroxyAMglucuronide; parent ion, m/z 508) in rat bile. (From Maggs et al. 2000). Reprinted with permission of The American Society for Pharmacology and Experimental Therapeutics.

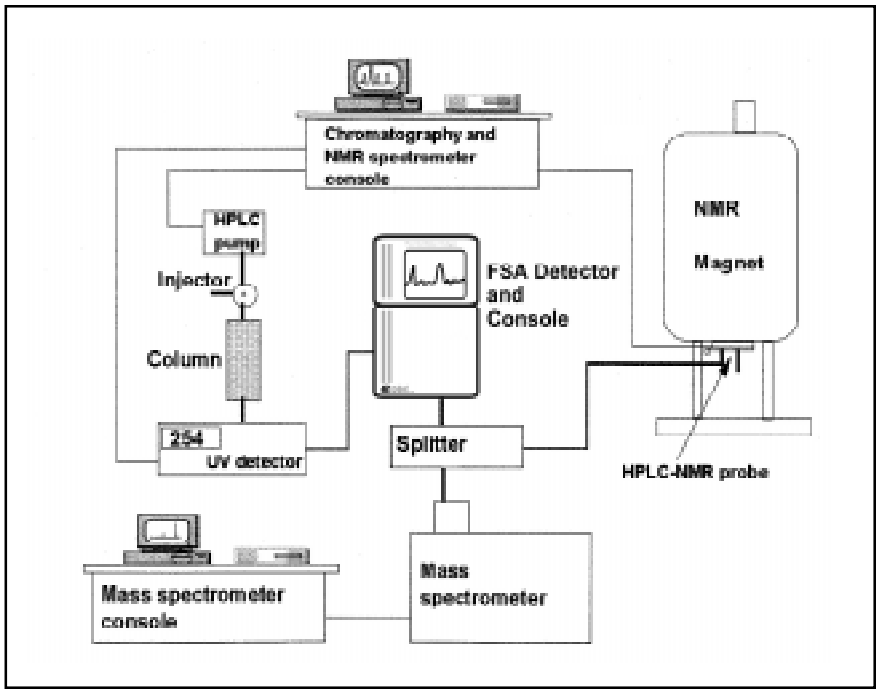

Figure 5.
Instrumental setup of the HPLC-UV-FSA-NMR-MS apparatus. (Modified from Hansen et al., 1999). Reprinted with permission. Copyright (1999) American Chemical Society.

*Footnote:
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