Flow Scintillation Analysis Application Note

Flow Scintillation Analyzer (FSA) Interfaced with the HPLC and Nuclear Magnetic Resonance (NMR) Spectrometer "A State-of-the-Art Application of the Radiomatic™ FSA"

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Introduction

Flow scintillation analysis is commonly used to quantify the radioisotope label on organic compounds such as biochemicals, drugs, and metabolites separated from complex mixtures by High Performance Liquid Chromatography (HPLC). The subsequent task of determining the molecular structure of the separated substances can be formidable. Traditional methods of structure determination involve collecting the HPLC separated fractions that correspond to activity peaks measured by the flow scintillation analyzer. The collected fractions are then isolated, further purified, and then submitted to spectroscopic methods of analysis such as mass spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy. Both MS and NMR methods provide complementary information that can be used to derive a molecular structure. Mass spectrometry can provide the molecular weight, molecular formula, and structure from ion fragmentation patterns, while NMR can provide additional important structural information including the spatial orientation of atoms in the molecular structure. A good example of this can be taken from early work of the author concerning the mass and NMR spectra of the inositol diastereomers, all of which produce virtually identical electron-impact mass spectra, but different NMR spectra (L'Annunziata, 1970 and L'Annunziata and Fuller, 1971 and 1976). A new and increasingly popular approach to drug metabolism and natural product studies involves the direct measurement of the NMR spectra of compounds directly off the HPLC column obviating the need for compound isolation. This application note will describe new developments in linking the Radiomatic flow scintillation analyzer from the HPLC to the NMR spectrometer to provide on-line (*in situ*) molecular structure analysis of radioisotope-labeled compounds.

Principle of NMR Spectroscopy

NMR Spectroscopy has been used to derive the molecular structure of organic compounds from the magnetic properties of the atomic nuclei (e.g., ^{1}H and ^{13}C) and the surrounding molecular electrons since the first commercial NMR spectrometer appeared in 1960. Nuclei of certain atoms of odd mass such as ^{1}H and ^{13}C, or even mass and odd charge have a net charge and a spin. The spinning charge of the nucleus creates a magnetic dipole (μ). If one places the spinning proton nuclei, which are a component of most organic compounds, in a magnetic field (*H*) the axis of the magnetic dipoles of these nuclei will precess at an angle (θ) with respect to the magnetic field axis as illustrated in Figure 1.

The precession of the nuclei with respect to the applied magnetic field axis occurs somewhat like the way a spinning top precesses under the force of the Earth's gravitational field. The angular velocity of this precession is a function of the strength of the applied magnetic field (*H*) and the effects of *shielding* caused by spinning electrons in the environment of the proton nuclei. While under the forces of a stable magnetic field (*H*) of the NMR spectrometer the proton nuclei are irradiated with radio frequency energy tunable over a narrow range. When the variable frequency is attuned to the recessional angular velocity of a given proton nucleus of a molecule, the two frequencies are in *resonance*. The applied energy at this resonance frequency is absorbed by the proton nucleus, and the nucleus is caused to flip or become aligned against the applied magnetic field (*H*). The energy absorbed by the proton nucleus that causes it to reach the higher energy spin state (i.e., flip) is the energy measured by the NMR spectrometer.

Figure 1. *A spinning proton nucleus in (a) the absence and (b) the presence of an externally applied magnetic field, H. (L'Annunziata, 1984) Reprinted with permission of Academic Press, Inc. San Diego, CA.*

Fortunately, in NMR spectroscopy, the resonance absorption by proton nuclei is complicated by the shielding effect of electron clouds of varying densities in the environment of organic molecules. The electron cloud surrounding a nucleus also has charge and spin, and therefore produce its own characteristic magnetic field, which apposes or shields the externally applied field. The degree of *shielding* is a function of the electron cloud density, which will differ from nucleus-to-nucleus in the organic molecule, because of the differing electronegativities of neighboring atoms. Therefore, protons in a molecule will absorb different resonance frequencies depending on their location in the molecule. This effect is referred to as the *chemical shift*. A proton, which is highly shielded, absorbs at a lower
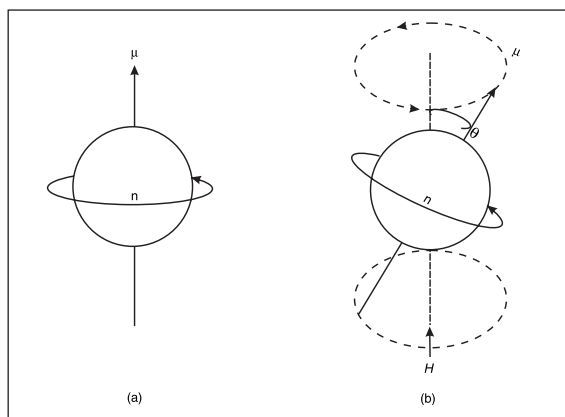
resonance frequency than a proton with reduced shielding. The presence of atoms of differing degrees of electronegativity (electron-withdrawing ability) in molecules as well as the differing three-dimensional orientation of atoms within molecules will cause a wide spectrum of shielding effects on neighboring protons. This gives rise to a wide spectrum of resonance absorption frequencies for protons depending on the structural group to which the protons are attached, their neighboring atoms, and their spatial orientation in the molecule. Therefore, the differing resonance absorption frequencies or chemical shifts of protons in NMR spectroscopy provide an absorption spectrum, which serves as a means for identifying chemical groups and their positions in organic molecules.

The chemical shift of a particular proton nucleus in a molecule is recorded with respect to the chemical shift of the protons on the reference molecule, tetramethylsilane or $(CH_3)_4Si$ most often referred to as TMS. The difference in chemical shifts of a proton or group of protons in a molecule with respect to that of TMS is recorded and calculated in units of Hz, whereas the magnitude of the applied frequency is in the order of magnitude of MHz, a million-fold greater. The difference in chemical shift of a proton nucleus with respect to that of TMS in Hz is divided by the applied frequency in MHz to record chemical shifts in convenient units of parts per million (ppm).

HPLC-FSA-NMR System

To maximize productivity, the trend is to analyze samples as fast as possible with as much automation that current technology will permit. This has led to recent advances in metabolism studies where the molecular structure of isotope-labeled metabolites must be determined. A major and relatively recent advance in this field has been the direct linking of the NMR spectrometer to the high performance liquid chromatograph (HPLC). Several papers on this technology serve as excellent examples (Bailey, *et al.*, 2000, Hansen, *et al.*, 1999, Shockcor, *et al.*, 1996, and Smith, *et al.*, 1999).

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 ^3H, ^{14}C, ^{33}P, ^{32}P, etc., is administered in a known quantity to a test animal and the metabolites separated by HPLC, the percentage of the total radioactivity administered is automatically measured by the FSA prior to NMR spectroscopy. This is illustrated later in this application note with data taken from the work of Sweeney, *et al.*, (2000). Consequently, the use of FSA (Flow Scintillation Analysis) prior to NMR spectroscopy 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) 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.

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 in the NMR spectrometer. The 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 NMR spectroscopic analysis. This will allow the researcher using HPLC-FSA-NMR to accurately stop the flow and capture the HPLC peak of interest in the NMR flow probe for molecular structure analysis. The FSA is connected between the UV and NMR if using a heterogeneous (solid) flow cell as illustrated in Figure 2. If a homogeneous (liquid) flow cell is used, the flow is split to both the FSA and NMR. The heterogeneous flow cell uses a solid scintillant detector of radioisotope label (e.g., ^3H, ^{14}C) providing full recoveries of the HPLC eluate for subsequent NMR analysis.

A popular heterogeneous flow cell for the FSA utilizes SolarScint™ 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 NMR spectroscopy (i.e., no effluent splitting). The homogeneous flow cell arrangement requires HPLC effluent splitting, because scintillation cocktail is mixed with effluent for radioisotope analysis. The latter homogeneous flow cell setup is most appropriate for ^3H analysis with detection efficiencies of up to 45% depending on the quench level of HPLC solvents.
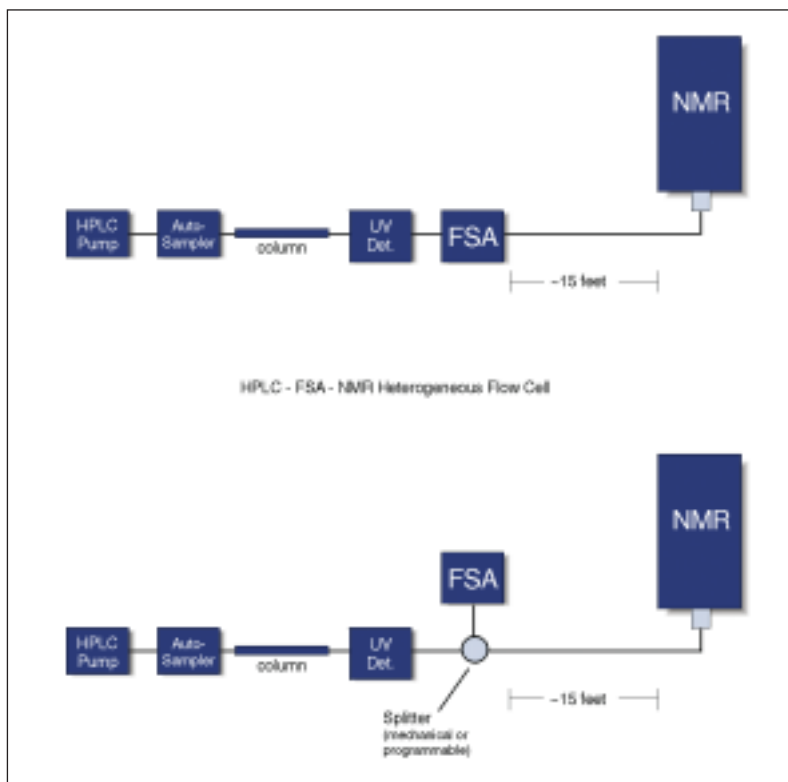
Figure 2.
Instrumental setup for the use of a Flow Scintillation Analyzer (FSA) with on line HPLC-NMR. A heterogeneous flow cell in the FSA (upper) allows for the entire effluent from the HPLC to continue on to the NMR spectrometer, whereas a homogeneous flow cell would require stream splitting prior to NMR spectroscopy. The relatively long distance (~15 feet) between the HPLC-UV-FSA instrumentation and the NMR is to protect the instrumentation from interferences caused by the magnetic field of the NMR.

A specially designed flow probe is inserted into the NMR sample chamber. The probe is constructed to permit the sample to flow into the NMR spectrometer and the resonance spectra obtained while either flowing through, or more commonly stopped and analyzed for a required period of time. The probe placed in the bore of the magnet holds the sample with a commonly employed cell volume of 120 μL. It contains the antennae for sample radio frequency energy irradiation and the receipt of the weak radio frequency resonance signal. A stop-flow mode is commonly employed for measurement of the NMR spectra, because of the low sample concentrations in the HPLC peaks. Suitable NMR spectra are obtained with samples as small as 1 μg (or even sub-microgram) depending on sample molecular weight and analysis time (Beery, 2000 and Silva-Elipe, 2000). Sample analysis times can vary from 1–2 hours to 1–2 days. Stop-flow NMR measurements of single peaks of the liquid chromatogram are governed by the signal from the UV absorbance detector or the signal from the FSA radioactivity detector. A signal from the radioactivity detector also confirms a metabolite of an isotope-labeled parent compound and quantifies the isotope label in that metabolite, while peaks observed from the UV detector, that do not coincide with radioisotope peaks can be ignored. The FSA detector, therefore, can be used to not only trigger stop-flow for NMR analysis and save valuable experimental time by permitting the researcher to ignore unlabeled UV peaks, but also provides valuable data for metabolic studies.

HPLC-FSA-NMR Representative Data

The application of the FSA in HPLC-NMR setups for the chromatographic purification, radioactivity label analysis, and molecular structure analysis of isotope-labeled metabolites can be found in numerous recent reports in the scientific journals. Only a few will be cited in this note (Dockens, *et al.*, 2000, Kumar, *et al.*, 1999, Maurizis, *et al.*, 1998, Paulson, *et al.*, 2000, Scarfe, *et al.*, 2000, Sweeny, *et al.*, 2000, and Vickers, *et al.*, 1998). Some researchers will use the stop-flow method described above, where the signal of a liquid chromatogram peak from the FSA radioactivity detector or UV detector will trigger the stop-flow needed for *in situ* NMR spectroscopy in the HPLC eluate. Others will utilize the same FSA or UV signal to collect the entire peak in a suitable vial and then submit the sample to further purification prior to NMR analysis in a suitable solvent. A representative example of the application of HPLC-FSA and the subsequent NMR spectroscopic results obtained will be cited subsequently.

In a study on the metabolism of the prodrug oseltamivir of the influenza neuramidase inhibitor GS-4071, Sweeny, *et al.*, (2000), administered oral doses of [^{14}C]oseltamivir to rats. Metabolites in rat urine, plasma, liver, and lung were separated by HPLC and on-line radioactivity of metabolite liquid chromatogram peaks were determined with a Radiomatic FSA with FLO-ONE® for Windows®. The elution times of the metabolites were determined with the Radiomatic FSA and via UV absorbance. A representative radiochromatogram printout from the Radiomatic FSA obtained from the urine fraction is illustrated in Figure 3.

The FLO-ONE® for Windows used with the FSA in this work is a comprehensive radio-HPLC workstation software package developed to exploit the graphical user interface and multi-tasking capabilities offered by Windows. The liquid chromatogram peak labeled GS4104 is that of the radioisotope-labeled parent compound [^{14}C]oseltamivir. The peak labeled GS4071 is the influenza inhibitor and peaks labeled M1 to M5 are metabolites, some of which are illustrated in the metabolic sequence in Figure 4. The FLO-ONE® software provided quantitative analysis of the metabolites as these were eluted from the HPLC column.

The molecular structure of metabolites were derived from evidence provided by mass and NMR spectral data. Among the metabolites purified by radio-HPLC, the metabolite peak labeled M3 in the Radiomatic FSA printout serves as an excellent example of the structural derivation from NMR data alone. By comparing the ^{1}H-NMR resonance assignments (i.e., chemi-

Figure 3. *Representative radiochromatogram of rat urine after a single oral dose of [^{14}C]oseltamivir. A FSA with FLO-ONE® for Windows was used as the detector. (From Sweeny, et al., 2000). Reprinted with permission of The American Society for Pharmacology and Experimental Therapeutics*.*

cal shifts in ppm) for the oseltamivir parent compound to the assignments for the M3 metabolite, the structure for the M3 peak from the Radiomatic FSA could easily be deduced as that (P)-ϖ-carboxylic acid oseltamivir illustrated in Figure 4. The ^{1}H-NMR resonance assignments taken from the NMR spectra are provided in Table 1 at the end of this application note. This work of Sweeny, *et al.*, (2000), clearly demonstrated the power of the use of FSA to monitor the presence and quantitative data for the amounts of radioisotope labeled metabolites in HPLC effluent prior to NMR molecular structure analysis.
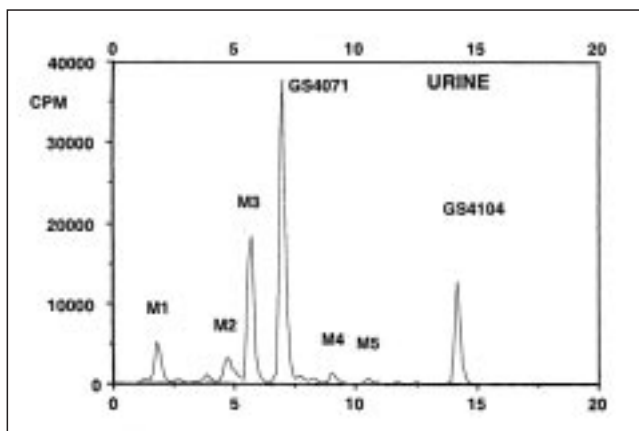
Future Trends

Several researchers are already using HPLC-UV-FSA-NMR-MS instrumentation. For on-line spectroscopic analysis of natural products, Bailey, *et al.*, (2000), Hansen, *et al.*, (1999), and Shockcor, *et al.*, (1996) split the HPLC efflu-ent in the proportions of 95% to the NMR spectrometer and the remaining 5% of the effluent to the mass spec-trometer 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, purifi-cation and subsequent spectroscopy are undertaken.
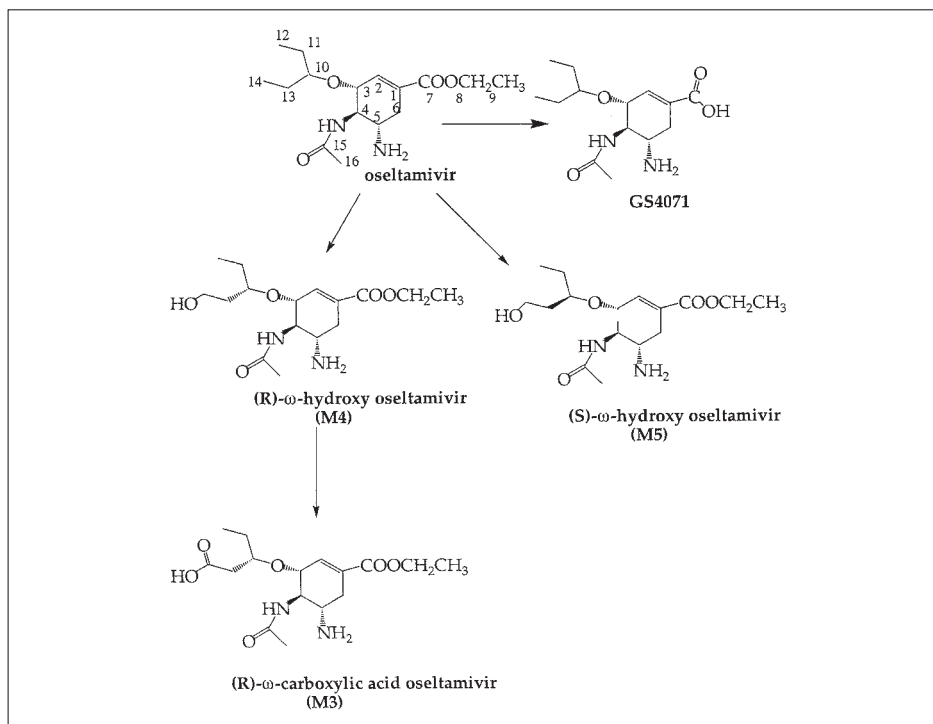
Figure 4. *Scheme of oseltamivir metabolism in the rat. (From Sweeny, et al., 2000). Reprinted with permission of The American Society for Pharmacology and Experimental Therapeutics*.*
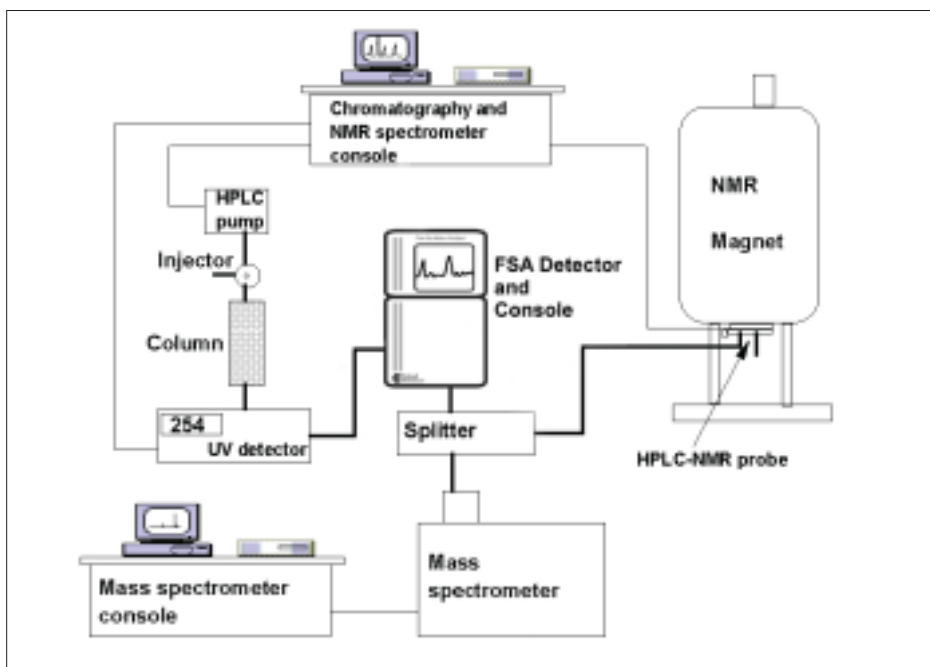

Figure 5. *Instrumental setup of the HPLC-UV-FSA-NMR-MS apparatus. (Modified from Hansen, et al., 1999). Reprinted with permission. Copyright (1999) American Chemical Society.*

Assignment	Oseltamivir		M3	
	Peak	J	Peak	J
	ppm	Hz	ppm	Hz
CH at C-2	6.87 (s)		6.87 (s)	
CH at C-3	4.36 (d)	8.9	4.36 (d)	8.6
CH$_2$ at C-8	4.27 (q)	7.3	4.27(q)	7.3
CH at C-4	4.06 (q)	11.6	4.06 (t)	10.0
CH at C-10	3.60 (m)		4.00 (m)	
CH at C-5	3.59 (m)		3.60 (m)	
CH$_a$ at C-6	3.00 (q)		3.00 (m)	
CH$_b$ at C-6	2.53 (m)		2.56 (m)	2.0
CH$_a$ at C-13	1.59 (m)		2.53	
CH$_b$ at C-13	1.57 (m)		2.40	
CH$_3$ at C-16	2.10 (s)		2.09 (s)	
CH$_2$ at C-11	1.57 (m)		1.60 (m)	
CH$_3$ at C-9	1.30 (t)	7.3	1.31 (t)	7.3
CH$_3$ at C-12	0.90 (t)	7.3	0.86 (t)	7.3
CH$_3$ at C-14	0.87 (t)			

Table 1[a]. *^{1}H-NMR Assignments (ppm), Multiplicities, and Coupling Constants (Hz) for Oseltamivir and M3 in Deuterium Oxide (s), singlet; (d) doublet; (t) triplet; (q) quadruplet; (m) multiplet.*

[a]   (From Sweeny, *et al.*, 2000). Reprinted with permission of The American Society for Pharmacology and Experimental Therapeutics*.

*   Footnote: The statements and opinions contained in the articles of the Journal *Drug Metabolism and Disposition* are solely those of the individual authors and contributors and not of the American Society for Pharmacology and Experimental Therapeutics. The American Society for Pharmacology and Experimental Therapeutics disclaims responsibility for any injury to persons or property resulting from any ideas or products referred to in the articles.

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