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Journal of Chromatography B, 826 (2005) 160–168
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Selective photo-reduction of *N*-nitroamines combined with micellar electrokinetic chromatography and laser fluorimetric detection

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Received 23 May 2005; accepted 18 August 2005

Abstract

N-nitroso compounds (NOC) are potent carcinogens. Reliable methods for the analysis of volatile carcinogenic NOC are well established; however selective and sensitive methods for routine analysis of thermally unstable, ionic or non-volatile NOC are still needed. For this purpose, a method based on micellar electrokinetic chromatography (MEKC) with laser induced fluorescence (LIF) detection is described for the simultaneous determination of a broad range of *N*-nitroso compounds. In this procedure, the nitroso group is photolytically cleaved from the NOC to yield the corresponding amine. The amines are then derivatized with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), identified and quantified using MEKC-LIF. For the standard mixture of NOC, this method has good sensitivity and a large dynamic range. The detection limit provided by the method is 9 ppb for *N*-nitrosopyrrolidine.
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Keywords: Nitrisamines; Laser fluorimeric detection; Carcinogen

1. Introduction

N-nitroso compounds (NOC) are potent carcinogens. More than three hundred nitrosamines have been tested for animal carcinogenicity and of these, 90% were found to be animal carcinogens and by extrapolation human carcinogens as well. [1] The ease of formation of *N*-nitroso compounds and the ubiquity of the precursors give *N*-nitroso compounds a potentially wide range of occurrences. Reducing human exposure to these carcinogenic compounds depends on the ability to accurately detect their presence in the environment and in food products. Development of reliable methods for the analysis of volatile *N*-nitroso compounds and methods for the analysis of low molecular weight NOC has aided in understanding the sources of NOC formation and has lead to the discovery of these compounds in the environment and food products. However, there remains a wide variety of NOC, specifically large nonvolatile, polar, ionic and thermally unstable NOC that are not monitored due to lack of appropriate analytical methodologies. Until suitable methods for the determination of total NOC content in diets are developed, their presence cannot be correctly ascertained. This limits the reliability of epidemiological studies that associate the total NOC content in diets with occurrence of some human cancers. Without suitable methods to determine the presence of non-volatile NOC, the ability to assess the total human exposure to NOC remains severely limited [2].

For the analysis of non-volatile nitrosamines, several methods for high performance liquid chromatographic separations of nitrosamines have been reported. Pre-column and post column derivatization has been used in the past to enable the selectivity and sensitivity needed for trace analysis in environmental samples and foodstuffs. High-pressure liquid chromatography with a thermal energy analyzer (HPLC–TEA) [3] and high-pressure liquid chromatography with a laser induced fluorescence detector (HPLC–LIF) are the most predominate of these techniques [4]. HPLC separations of nitrosamines have been reported using fluorescence detection after chemically cleaving the *N*-nitroso group forming the corresponding amine [5].

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doi:10.1016/j.jchromb.2005.08.019

This work demonstrates that micellar electrokinetic capillary chromatography (MEKC) with laser-induced fluorescence detection (LIFD) is a viable means for the analysis of NOC. A two-step process is described that involves photolysis of the nitrosamine to form the less hazardous corresponding amine followed by derivatizing the amine with 4-chloro-7-nitrobenzofuran (NBD-Cl) to yield a fluorescent product. The method is been demonstrated on a certified, commercially available volatile NOC standard. Since the method described does not require significant heating and permits separation and detection in the condensed phase, it is amenable to both volatile and non-volatile NOC. In addition, for those classes of *N*-nitroso compounds that can be analyzed using current techniques, this method may offer significant advantages over existing methods. One advantage of the method is a greatly simplified sample preparation. Currently, the most commonly used detector for NOC determination is the thermal energy analyzer (TEA). The TEA requires extensive sample conditioning prior to injection, consisting of distillations, solvent extraction and preconcentration. These steps are necessary to remove polar and inorganic salts that foul the detector. The method introduced here does not use extensive sample preparation procedures, which eliminates the loss of volatile nitrosamines and artificial formation of nitrosamines in the analytical procedure. The stability of the MEKC–LIFD system circumvents the need for sample purification and the sensitivity of the fluorescence detection eliminates the need for preconcentration for most samples. The method also possesses a high degree of selectivity, to which MEKC and fluorimetric detection both contribute.

Fluorescence detection contributes to selectivity, since only those molecules that both absorb at the chosen excitation wavelength and fluoresce at the chosen emission wavelength yield a signal. However, the main advantage of LIFD results from its unsurpassed sensitivity. The directionality of laser emission is well suited to capillary techniques. However, commercial electropherographs with laser fluorimetric detection are costly. A fluorescence cell has been designed and constructed that is inexpensive, simple and does not require the use of micro-positioners or expensive optics. Another advantage of the method is the ability to develop the electrophoretic separation without the use of the actual *N*-nitroso compounds, making the development safer for the experimenter. The first step in the analysis involves selective cleavage of the *N*-nitroso group to the corresponding amine. From that point on, the experimenter works with non-carcinogenic material. Optimization of the separation conditions was developed and tested without the use of hazardous standards. The presence of a new NOC may be identified by using the corresponding amine standard. Another advantage of MEKC–LIFD is that analysis may be performed on very small volumes of sample. Thus, research involving living organisms may require fewer subjects and be less traumatic to the subjects employed. Small sample volumes are an important aspect necessary to make in vivo sampling possible. A method applicable to endogenously formed non-volatile and thermally labile NOC such as *N*-nitroso proteins and peptides would be of great importance to the understanding of biogenic NOC production as well as aiding in the development of strategies for blocking it. Due to the derivitization step, quantitation of NOCs in "real samples" would best be performed using the method of standard additions [6].

2. Experimental

2.1. Reagents and materials

All materials were obtained from commercial suppliers and used without further purification, unless specifically noted. Analytical reagent grade chemicals were used along with deionized water to prepare solutions. Standard nitrosamines and amines were purchased from Aldrich (Milwaukee, WI, USA). The Nitrosamine mixture was an Environmental Calibration Standard used for EPA method 8270. This standard solution, originally 2000 mg/L of each NOC in methylene chloride, was diluted with an appropriate amount of 30% methanol solution to obtain target concentrations. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was supplied by Pfaltz and Bauer (Stanford, CT, USA). Buffer solutions were prepared using sodium dodecyl sulfate (SDS) from Sigma Chemical Co. (St. Louis, MO, USA) and boric acid from J.T. Baker (Phillipsburg, NJ, USA). The pH was adjusted using 1.0 M sodium hydroxide.

Diethylamine (DEA) was purified by fractional distillation using a Vigreux column and a cow collection flask. 10 mL of DEA was slowly heated in an oil bath to 70 °C. The initially yellow DEA, was distilled to give a clear colorless liquid. NBD-Cl was by re-crystallized from ethanol and water. NBD-Cl in the amount of 0.9 g was added to a 250 mL Erlenmeyer flask containing approximately 80 mL of water. The flask was then heated to 80 °C with stirring. The solution turned yellow, but less than half of the material dissolved. Approximately 50 mL of ethanol was added to complete the dissolution. The solution was then hot-filtered to remove undissolved solids. Upon cooling to room temperature, needle-shaped crystals slowly began to form. To speed the crystallization, the flask was put in an ice bath. On cooling, crystal plates also began to form. The crystals were cold-filtered and rinsed with ethanol. The recrystallization yielded about 50% of the original material.

2.2. Instrumentation

MEKC experiments were performed using an Isco Capillary Electropherograph Model 3850 (Lincoln, NE), equipped with a UV absorbance detector and a vacuum/electromigration injection accessory. The electropherograph employs a fan for temperature control. The configuration employed is diagrammed in Fig. 1. The 488 nm line from a 20 mW American Model 60× argon-ion laser (MWK Industries, Corona, CA, USA) operating at approximately 5 mW was chopped at 240 Hz with a locally fabricated chopper and focused into a 125 μm optical fiber for fluorescence excitation. The 125 μm excitation fiber and the 400 μm collection fiber were positioned flush to the separation capillary at a 90° angle. The 400 μm collection fiber terminated into a 540 nm interference filter (Esco Products, Oak Ridge, NJ, USA) in line with a Model HNF-488-1.0 holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI, USA) and finally,

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