Journal of Chromatography A, 1490 (2017) 80–88

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Full length article

Analyzing complex mixtures of drug-like molecules: Ion mobility as an adjunct to existing liquid chromatography-(tandem) mass spectrometry methods

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ARTICLE INFO

Article history:
Received 29 August 2016
Received in revised form 24 January 2017
Accepted 9 February 2017
Available online 14 February 2017

Keywords:
LC–MS
Tandem MS
Ion mobility
Isomer separation
Protomers
Melphalan

ABSTRACT

The use of traveling wave ion mobility mass spectrometry (TWIMS) is evaluated in conjunction with, and as a possible alternative to, conventional LC–MS(/MS) methods for the separation and characterization of drug-like compounds and metabolites. As a model system we use an *in vitro* incubation mixture of the chemotherapeutic agent melphalan, which results in more than ten closely related hydrolysis products and chain-like oligomers.

Ion mobility as a filtering tool results in the separation of ions of interest from interfering ions, based on charge state and shape/size. Different classes of chemical compounds often display different mobilities even if they show the same LC behavior – thereby providing an orthogonal separation dimension. Small molecules with identical or similar *m/z* that only differ in shape/size (e.g. isomers and isobars, monomers/dimers) can also be distinguished using ion mobility. Similar to retention times and mass-to-charge ratios, drift times are analyte-dependent and can be used as an additional identifier.

We find that the compound melphalan shows two different drift times due to the formation of gas-phase charge isomers (protomers). The occurrence of protomers has important implications for ion mobility characterization of such analytes, and also for the interpretation of their fragmentation behavior (CID) in the gas phase.

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1. Introduction

LC–MS/MS is one of the most powerful and favored analytical techniques for the analysis of small molecules in complex matrices. Nonetheless, the analysis of samples containing co-eluting compounds and analytes with similar or identical MS properties (mass, fragmentation and/or isotope patterns) remains a frequent challenge. One of the breakthrough analytical technologies in the last decade is ion mobility-mass spectrometry (IM-MS) [1–3]. As a gas-phase separation technique, IM characterizes ions based on their collision cross-section (CCS; a parameter related to the ion's rotationally averaged size, shape, total charge and charge distribution). An acceleration voltage is applied along the ion's flight path through the drift cell filled with neutral buffer gas [4]. Due to friction, i.e. collisions with the gas molecules, the ions reach a characteristic velocity, and the time it takes for an ion to pass through the cell is called the drift time (t_d). The type of drift gas used, typically He or N_2, has a significant effect on the resolving power of ion mobility [5,6]. Moreover, the use of polarizable drift gases (e.g. N_2 or CO_2) can lead to separation of charge site isomers, i.e. (de)protomers, of an otherwise identical analyte molecule (see below).

Ion mobility itself has proven to be effective e.g. for detection of narcotics and explosives [7,8]. Although its hyphenation with MS in commercial instrumentation is a more recent development, it has already created new possibilities for the characterization of some classes of small molecules and metabolites [9–11]. Specific examples are the separation of isomeric disaccharides [12–14] and metal(-ligand) complexes [15–17] or the characterization of carbohydrate, glycan and lipid structures [18–23]. Particular interest recently focused on the phenomenon of alternative charge sites in small molecules that are observed in the gas phase, i.e. the observation of protonation site isomers (protomers) [24–26]. The routine use as a separation technique is however still in its infancy, and the

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http://dx.doi.org/10.1016/j.chroma.2017.02.015
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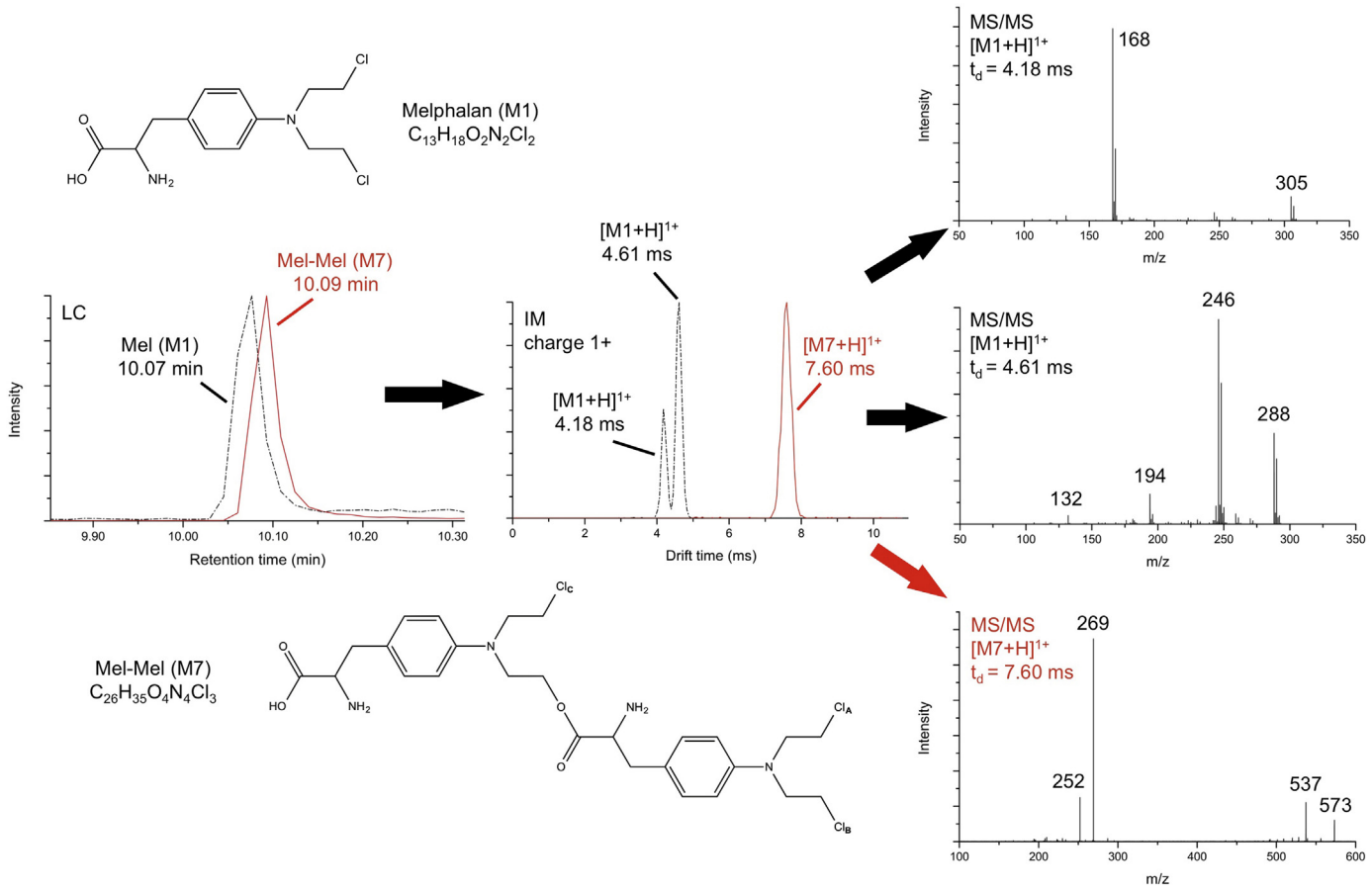

Fig. 1. Visualization of an LC-IM-MS/MS experiment. Data is acquired in multiple dimensions: LC, IM, MS and MS/MS. LC peak and drift time profile are displayed for melphalan (M1; *m/z* 305) and the dimer M7 (*m/z* 573; for compound labels see Table 1). For melphalan, two different drift times are observed and each one results in a different MS/MS spectrum. In the CID spectrum of the t_d = 4.61 ms species, the melphalan precursor ion is fully fragmented (no *m/z* 305 peak left; middle spectrum in right column).

added benefit (if any) of combining IM with LC–MS/MS has not been studied systematically. Here we utilize traveling wave ion mobility spectrometry (TWIMS), which has been described previously [27–29].

An important application of TWIMS in the context of complex samples is background filtering. Often samples contain unwanted additives such as salts, plasticizers or interfering analytes that hinder the detection of the compounds of interest. Even though ion suppression may still occur in the electrospray process, IM-MS can reveal specific analyte ions which are present in the spectra but difficult to discern, by separating them from interfering background ions based on their charge and shape/size [30,31]. Such filtering can also be performed post-acquisition, provided that the data were recorded in ion mobility mode [32].

Given that LC separations have a timescale of minutes and time-of-flight (TOF) detection occurs in microseconds, IM is easily integrated with its millisecond regime without affecting the timescale of the existing LC–MS/MS method, by adding an extra, orthogonal separation dimension. Relatively few studies have been published on the use of LC-IM-MS/MS so far [33–38], but this number is expected to increase considerably in the next years. While retention time (t_R), drift time (t_d) and mass-to-charge (*m/z*) provide key analytical determinants (e.g. polarity, shape/size and mass), the characterization of compounds solely based on these parameters could lead to incorrect assignment, and is often considered insufficient in practice. Fragmentation by MS/MS on the other hand is generally employed to obtain structural information [39,40], and we also investigate its use here together with ion mobility analysis. Recently IM-MS/MS has been used to enhance peptide detection [41], study the fragmentation patterns of drug metabolites [42] and (de-)protomers [24–26,43,44].

The present study focuses on the efficiency and versatility of ion mobility as an adjunct to LC–MS/MS methods, by using a model compound mixture with moderate analytical complexity. We previously investigated melphalan (L-phenylalanine mustard; see Fig. 1), an alkylating agent which is used in the treatment of cancer [45,46]. Several studies have been published discussing its (pharmaco)kinetics, binding to DNA and proteins [47–50], and its clinical applications. In aqueous solution, melphalan forms two major hydrolysis products [51,52] *in vitro*, mono- and dihydroxy melphalan, as well as chain-like oligomers [53]. In total, more than ten closely related degradation products were characterized before (Table 1). Understanding the complexity of these drug-related compounds is essential for our ability to accurately determine drug levels in patients, and particularly also for the quantitation of the active form of the drug in different sample matrices.

2. Materials and methods

Caution: melphalan, degradation products and oligomers are carcinogenic and should be handled with care.

2.1. Chemicals

Melphalan (Mel; min. 95%) and sodium formate (HPLC, > 99.0%) were purchased from Sigma-Aldrich (Bornem, Belgium). Acetonitrile (ACN; HPLC grade for gradient analysis) and formic acid (FA; 99+ %) were obtained from Acros (Geel, Belgium). Reverse osmosis

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