Journal of Chromatography B, 986–987 (2015) 135–142

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Development of sample clean up methods for the analysis of *Mycobacterium tuberculosis* methyl mycocerosate biomarkers in sputum extracts by gas chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 30 October 2014
Accepted 8 February 2015
Available online 16 February 2015

Keywords:
Cholesterol
Mycobacterium tuberculosis
Solid phase extraction
Molecularly imprinted polymers
Thermochemolysis
GC–MS

ABSTRACT

A proof of principle gas chromatography–mass spectrometry method is presented, in combination with clean up assays, aiming to improve the analysis of methyl mycocerosate tuberculosis biomarkers from sputum. Methyl mycocerosates are generated from the transesterification of phthiocerol dimycocerosates (PDIMs), extracted in petroleum ether from sputum of tuberculosis suspect patients. When a high matrix background is present in the sputum extracts, the identification of the chromatographic peaks corresponding to the methyl derivatives of PDIMs analytes may be hindered by the closely eluting methyl ether of cholesterol, usually an abundant matrix constituent frequently present in sputum samples. The purification procedures involving solid phase extraction (SPE) based methods with both commercial Isolute-Florisil cartridges, and purpose designed molecularly imprinted polymeric materials (MIPs), resulted in cleaner chromatograms, while the mycocerosates are still present. The clean-up performed on solutions of PDIMs and cholesterol standards in petroleum ether show that, depending on the solvent mix and on the type of SPE used, the recovery of PDIMs is between 64 and 70%, whilst most of the cholesterol is removed from the system. When applied to petroleum ether extracts from representative sputum samples, the clean-up procedures resulted in recoveries of 36–68% for PDIMs, allowing some superior detection of the target analytes.

1. Introduction

Tuberculosis (TB) is an infectious disease that is still of increasing concern, with higher incidence in the underdeveloped countries, it represents a world scale problem, given the extensive international mobility of people in the 21st century [1], and its early diagnosis is crucial in reducing and stopping the disease from spreading [2].

The cell envelopes of *Mycobacterium tuberculosis* are rich in unusual lipids, not present in the mammalian tissue. These compounds are therefore considered as proper mycobacterial biomarkers that can enable the disease detection in fluids from infected patients.

Gas chromatographic–mass spectrometric (GC–MS) analysis, following the offline or online derivatization of lipids from the mycobacterial cell membrane, has been proven to be successful for the TB diagnosis by detecting *M. tuberculosis* biomarkers in sputum [3–7]. The 10-methyl octadecanoic acid biomarker, also known as tuberculostearic acid (TBSA), has been frequently analyzed by GC–MS in positive [3–6] and negative chemical ionization (NCI) modes [7], but it is almost ubiquitous among the members of mycolata class, and in other genera such as *Nocardia*. Furthermore, TBSA is dispersed in the cell envelope within a range of plasma membrane phospholipids and lipoglycans, and access to the full

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http://dx.doi.org/10.1016/j.jchromb.2015.02.010
1570-0232/

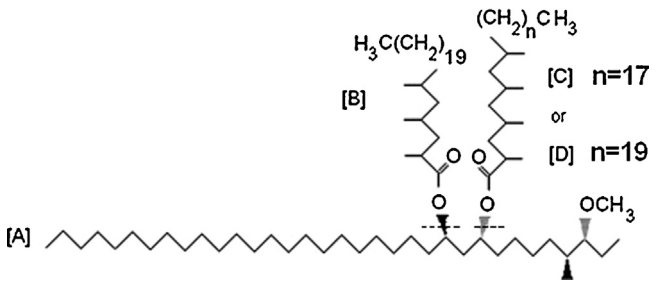

Fig. 1. The molecular structure of phthiocerol dimycocerosates (PDIMs), prior to thermochemolysis. The dotted lines show the sites of cleavage during the thermally assisted hydrolysis and methylation process.

content of TBSA requires its prior hydrolytic release by complicated chemical manipulations [3,4,6,7]. Direct analysis of TBSA by GC–MS was reported [5], following thermochemolysis of aliquots from sputum samples, but our experiments based on this procedure [8] resulted in poor sensitivity and specificity, owing to the TBSA ester co-elution with derivatives of other matrix compounds, such as the methyl ester of octadecanoic acid, which is usually present in excessive amounts in sputum.

There are other classes of lipids which can be immediately accessible for rapid analysis, using simple solvent extraction from culture or sputum sample deposits. Our study involves the more accessible class, the family of phthiocerol dimycocerosates (PDIMs) shown in Fig. 1. They are stable, high molecular weight hydrophobic waxes of around a 90 carbon backbone, which are highly resistant to hydrolysis, and hence will survive the usual procedures used to sterilize infected materials isolated from patients. The mycocerosate components are only present in a limited number of mycobacteria (*Mycobacterium microti*, *Mycobacterium bovis*, *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium ulcerans* and *Mycobacterium leprae*) [9–12]. The methyl derivatives of mycocerosates from *M. tuberculosis* give characteristic doublet peaks with dominant C_{29}, C_{30}, and C_{32} components. In an early study by Larsson, 5-days-old cultures of sputum specimens were shown by GC–MS to have C_{32} mycocerosates [6]. Negative-ion chemical ionization GC–MS methods developed for sensitive detection of mycobacterial mycocerosates [9], has been used to detect mycocerosate biomarkers for ancient tuberculosis in a skeletal collection [13].

Our work focuses on PDIMs as excellent *M. tuberculosis* biomarkers. Following apolar lipid extraction from sputum, PDIMs were submitted to thermally assisted hydrolysis and methylation (THM) in the programmed temperature vaporizer (PTV) inlet, and the resulting methyl mycocerosates, shown in Fig. 2, were then analyzed by GC–MS. The initial THM-GC–MS method development [14] and its application to a batch of positive and negative real sputum samples [15] were presented in our earlier publications, which focused on the overall method performance without reporting and discussing the particular results obtained in certain sputum sample extracts with a high level of matrix compounds. Blind analysis of the PE extracts of 400+ sputum specimens [15], using our THM-GC–MS method gave 64.9% sensitivity and 76.2% specificity, and it was noted that other components of sputum, such as cholesterol, may hinder the analysis. The presence of matrix peaks in the retention time region of the analytes and a high baseline rendered difficult the assignment of the target doublet peaks, when a low target signal was hidden by abundant background peaks. Derivatized matrix compounds build up in the inlet, which eventually leads to active sites and may compromise subsequent runs. This also impacted on the column life time, causing frequent column overloading and even column blockages, the capillary column needed trimming and/or replacement. Both the PTV inlet and the

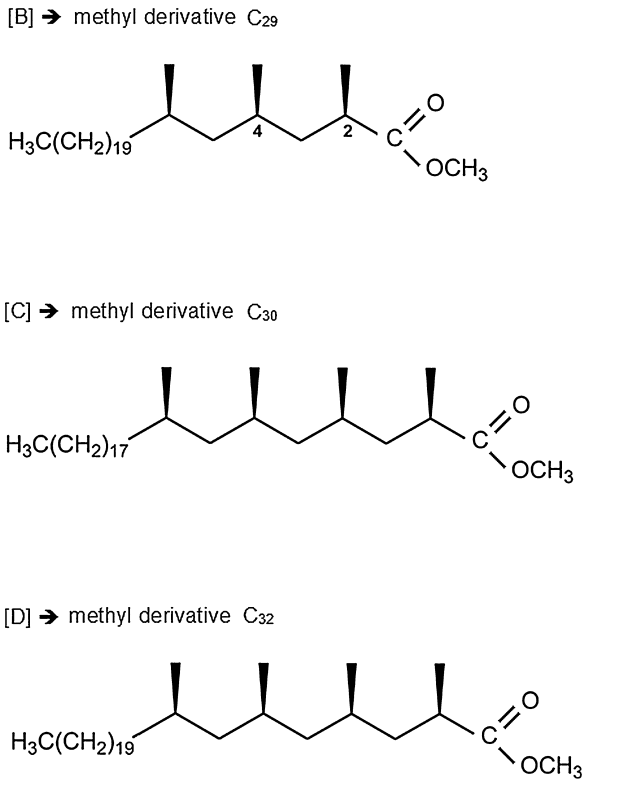

Fig. 2. Molecular structures of the target analytes resulted from the transesterification of the PDIM moieties (B)–(D) as shown in Fig. 1, via thermally assisted hydrolysis and methylation in the PTV inlet.

EIMS ionization source required cleaning on a regular basis, which is not ideal for a routine method. It is therefore desirable to extract PDIMs from the sputum sample, and to filter the extract in order to purify and concentrate them prior to analysis.

In the present work, a proof of principle clean up method was developed, aiming for a maximum recovery of PDIMs and minimum collection of cholesterol from a stock solution of PDIMs and cholesterol standards in PE. The lipid extraction from sputum samples was performed with a combination of apolar petroleum ether (PE) and an immiscible polar solvent (methanol), applying a modified Dobson protocol [10]. Four PE extracts of positive sputum samples were selected that were previously [15] found to have high amounts of cholesterol. These were then passed through different solid phase extraction (SPE) materials, both commercial and molecularly imprinted polymers (MIPs) cartridges. Molecular imprinting is performed by producing a polymeric matrix, containing complementary residues, in the presence of the target molecule [16], in this case cholesterol. After formation, the original template is removed, leaving cavities that are complementary to the shape and chemical profile of the template, only allowing specific recognition and rebinding.

2. Experimental

2.1. Molecularly imprinted polymer synthesis

Methacrylic acid (MAA), ethyleneglycol dimethacrylate (EGDMA) and azo-N,N′-diisobutyronitrile (AIBN); and all solvents (HPLC grade) were purchased from Sigma Aldrich, (Dorset, UK).

Cholesterol is a favoured template for imprinting studies and has been imprinted by several different researchers [17–19]. A generic MAA/EGDMA methodology was used as proof-of-principle,