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Towards a New Approach for Fast Diagnosis of Tuberculosis using Gas Chromatography-Mass Spectrometry

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Summary

Tuberculosis (TB) remains a major problem in public health worldwide. According to the World Health Organization an estimated 8.6 million people developed TB and 1.3 million died from this disease in 2012. Despite persistent efforts over the last 125 years to develop a simple tool for the rapid diagnosis of tuberculosis, the ideal method has proven elusive. It is clear that GC–MS offers considerable advantages for the analysis of complex biological samples. In this thesis we discuss two main areas in which progress is sought. The first is the discovery of novel biomarkers for diagnosis of tuberculosis using GC−MS and chemometrics. The second is the development of an affordable, accurate and 'simple to use' point of care test for diagnosing tuberculosis using a small (trans)-portable or hand-held device based on GC technique.

Chapter one of this thesis describes briefly the role of analytical chemistry and chemometrics in the analysis of complex samples. A brief overview of recent developments and remaining problems in the diagnosis of tuberculosis is also presented. It is then explained why biomarker discovery has become a strong desire in TB research. Finally it is explained how modern, highly powerful GC−MS techniques can be applied for identification and quantification of specific biomarkers for TB in real specimens from suspected patients.

Chapter two of the thesis briefly summarizes the current status of tuberculosis diagnostics research and future aspects in the use of GC–MS to search for novel biomarkers of MTB. Volatile biomarkers for TB in the headspace of bacterial culture samples or in samples of breath, serum or urine show little consistency in the various studies to date. Reproducibility is difficult; the impressive results found initially with a few patients are rarely repeatable when a larger sample series is tested. Break-down products derived from mycobacterial lipids show more consistency as biomarkers of tuberculosis in several studies. This approach offers promise for distinguishing MTB from NTM directly in sputum and does offer great potential towards a much simpler near-bedside test that could be widely used in resource-constrained countries.

Chapter three of the thesis describes a promising approach for the identification of novel biomarkers to differentiate MTB and NTM in early cultures using a fully automated procedure based on thermally-assisted hydrolysis and methylation followed by GC-MS (THM−GC−MS) and advanced chemometrics. We used early cultures of 15 MTB and 29 NTM strains from the Netherlands grown in

Middlebrook 7H9 liquid medium to build a classification model for distinguishing MTB from NTM. The final model performed with better than 95% accuracy in differentiation between MTB and NTM in early cultures using a combination of 20 different compounds. These biomarkers have been chemically identified using mass spectral interpretation. Some of these compounds have not been linked to tuberculosis before, others have been proposed previously as diagnostic biomarkers for this disease.

Chapter four of the thesis demonstrates the validation of the 20-compound model developed in chapter three using two independent sample sets, one consisting of 56 isolates (39 MTB and 17 NTM) from the Netherlands, the other comprising 103 isolates (91 MTB and 12 NTM) from Stellenbosch, Cape Town, South Africa. All the MTB strains in the 56 Dutch samples were correctly identified and the model had a sensitivity of 100% and a specificity of 94%. For the South African samples the model had a sensitivity of 88% and specificity of 100%. The model performed less good for the South African sample set compared to the Dutch samples. Based on these results, it is clear that for optimum performance of the model, the training set used to derive the model should consist of locally occurring strains and should contain a balanced numbers of MTB and NTM strains (preferably approximately 50%). To improve the sensitivity of the model for South African strains, whilst maintaining a high specificity, we have developed new decision-tree models that allow the differentiation of MTB from NTM using two different algorithms. One algorithm was constructed using visual inspection of the data. This algorithm enabled correct classification of all 103 samples from South Africa as well as of the 100 samples from the Netherlands, i.e. it gave 100% sensitivity and specificity. This decision-tree model was based on the use of a combination of eight potential biomarkers from the 20-compound list. Another tree was fitted using the Classification and Regression Tree (CART) method. The resulting model was extremely simple (only two compounds were included), yet highly accurate (99.5% accuracy). Although promising, more samples need to be classified to evaluate this algorithm.

Chapter five of the thesis focuses on the development of a clean-up procedure for the analysis of the 20 potential biomarkers for diagnosing tuberculosis directly in sputum from South Africa. The final method for the analysis consisted of a hexane/methanol/water extraction followed by a further solid phase extraction (SPE) clean up and finally THM–GC–MS. Based on the mycocerosate markers, the detection limit of the method was approximatively 1×10^4 bacteria spiked. This detection limit is lower than that of microscopy and therefore sufficient for early detection of MTB directly in sputum. Finally, 32 South African sputum samples from patients suspected of having tuberculosis were blindly

tested using the method developed. The results from our method show an excellent agreement with those obtained from Ziehl-Neelsen and culturing. More samples need to be tested to further evaluate the sensitivity and specificity of our combined sample preparation and analysis method. The results obtained show that the new extraction-SPE–THM–GC–MS method is promising for detection of MTB directly in suspected sputum samples and holds great potential for fully automating the whole procedure. The work currently proceeds towards the development of a miniaturized GC-based device for detection of MTB at the point of care.

Chapter six of the thesis describes the development of a miniaturized GC-based device for detection of potential biomarkers for tuberculosis in the field. The device consists of a GC inlet equipped by a PTV injector that is connected to a heated micro-GC column cartridge. The device showed a good performance in the separation of low boiling components; unfortunately however, severe peak splitting, the so-called “Christmas tree effect”, was observed for higher boiling components such as the higher alkanes or higher FAMEs (e.g. C16 and C18). The peak splitting is caused by a non-uniform oven temperature along the length of the column. Clearly further improvements to the device are needed to allow separation of the high-boiling components on our target list of 20 marker compounds.

Chapter seven of the thesis presents an interesting application of the novel sample preparation method developed in chapter five of this thesis. The hexane/methanol/water extraction procedure followed by SPE–THM–GC–MS developed for the analysis of sputum samples was here applied for detection of *Mycobacterium avium subspecies paratuberculosis* (MAP) in cow manure. The method proposes the use of a combination of 10 potential biomarkers for the detection of MAP. An algorithm allows the differentiation of infected cows and non-infected cows giving a sensitivity of 76% and a specificity of 80% for 31 cows. The results look promising; but more work needs to done to improve the accuracy of this diagnostic test.