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Thin-layer Chromatography–Nuclear Magnetic Resonance Spectroscopy – A Versatile Tool for Pharmaceutical and Natural Products Analysis

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Abstract: Thin-layer chromatography (TLC) is a mature and very established technique, frequently used in many fields of applications ranging from natural product analysis to chemical or pharmaceutical applications. The introduction of a commercially available TLC–MS interface was a major step complementing the ease of use of TLC with structural elucidation power of mass spectrometry (MS). The TLC-MS interface simplifies the workflow dramatically to gain structural information directly from TLC separations. This article describes the potential of TLC-nuclear magnetic resonance spectroscopy (NMR) utilizing the TLC-MS interface to straightforwardly characterize zones of interest by NMR spectroscopy with a focus on quantification of active pharmaceutical ingredients (API) in formulations and identification of active principles in plant extracts.

Keywords: Interfaces · Natural products · Nuclear magnetic resonance · Pharmaceutical analysis · Thin layer chromatography · TLC-MS · TLC-NMR

Introduction

Thin-layer chromatography is a robust, well established and mature separation technology which is beneficially used in many different fields of research. Ease of use, low in price and with an intrinsic possibility for parallel analysis of several samples make this technique an extremely attractive analytical tool in chemical, pharmaceutical, and natural product research.[1] However, this kind of planar chromatography suffers from relatively poor separation efficiency compared to ultra-high performance liquid chromatography (UHPLC).

The introduction of a commercial TLC–MS interface by CAMAG AG (Muttenz, CH) in 2009 combined the ancient thin-layer chromatographic separation technique with modern mass spectrometric methods.[2] This was a key development and major achievement that allowed the exploitation of the full potential of TLC for robust and fast parallel separation of samples in complex matrices with today's information-rich detection technologies like MS. Although the principle of the interfaces relies on a relative simple extraction procedure of a zone of interest directly on the plate, thus mimicking the conventional scratch and extract process, this device found widespread use in the TLC community.

In contrast, NMR spectroscopy has not found frequent use as a detector for TLC separations mainly due to sensitivity limitations and high instrument costs. However, a unique selling point for NMR spectroscopy can be found in its linear correlation of signal intensity with sample amount independent of the availability of an internal standard.[3] This makes TLC-NMR an ideal technology complementing TLC-MS to characterize and quantify natural products in plant extracts, API's in formulation or by-products of chemical synthesis.

Basic Principles

Quantitative NMR

The important feature of quantitative NMR (qNMR) is that signal response, unlike other methods such as chromatography, is strictly linear correlated to the number of spins in the NMR active volume. NMR signal intensities are therefore independent of the structure. Thus, a single reference substance can be used as standard for all other compounds to be determined. This makes NMR spectroscopy a versatile universal and quantitative detector providing accurate, precise, and reliable results.[4–6] For quantification, often a reference compound of known concentration is added to the sample as an internal standard. However, it is difficult to find a general standard since overlap with all resonances of a diversity of analytes must be avoided. To circumvent these NMR-related shortcomings several methods based on external standards have been discussed like ERETIC[3] (Electronic Reference To access In vivo Concentrations) or PULCON[7] (Pulse Length based CONcentration determination). These methods are based on the intrinsic spectrometer stability and linearity of NMR signal response to concentration. While PULCON was originally developed for the measurement of protein concentrations, it is also applicable for small molecules. The determination of concentrations of NMR samples with PULCON is easy, straightforward and efficient. Moreover it is easy to implement and can be used on all NMR spectrometers as it does not require any special hardware or software. This robust method delivers accurate and precise concentration measurements.

Experimental Setup

In order to investigate the use of NMR spectroscopy for quantification and characterization of TLC separated compounds, four different test substances were separated by TLC (Table 1). For the formulated drug Amiodarone Mepha, 100 mg of the ground tablet were dissolved in 25 mL dichloromethane, filtered through a 0.2 µm syringe filter and 5 µL were applied to the TLC plate. The TLC system consisted of a CAMAG Automatic TLC-Sampler 4, and a CAMAG ADC 2 development chamber. HPTLC Silica Gel F254 DC plates were developed with methanol and dried at 60 °C and 50 Torr before use. All samples solutions were sprayed in triplicates on TLC plates and allowed to develop for 50 mm. Zones of interest were extracted from the TLC plate with methanol into a transfer vial using the CAMAG TLC-MS interface. Solvent was removed by a stream of nitrogen and the sample was finally collected in 600 µl methanol-d_4 for subsequent NMR measurement on a 400 MHz Bruker Avance I instrument, equipped with a 5mm BBO probe and controlled by TOPSPIN 2.1. Standard 1D proton spectra with WET solvent suppression[8] were acquired to remove remaining solvent signals. For this 32768 data points were collected with

Table 1. Samples, separation methods and NMR integral range for all tested compounds

Substance	TLC Method	Rf	LOD (NMR) [µg]	Integral range [ppm]
Rutine	formic acid, ethylacetate, H_2O, ethylmethylketone (2.5:15:3:9 V/V)	0.32	2.3	1.04–0.94
Caffeic acid	formic acid, ethylacetate, H_2O, ethylmethylketone (2.5:15:3:9 V/V)	0.85	2.5	6.27–6.21
Chlorogenic acid	formic acid, ethylacetate, H_2O, ethylmethyl-ketone (2.5:15:3:9 V/V)	0.49	3.3	6.32–6.26
Amiodarone	formic acid, methanol, dichloromethane (5:10:85 V/V)	0.68	2.8	8.21–8.19
Amiodarone Mepha 200 tablet	formic acid, methanol, dichloromethane (5:10:85 V/V)	0.68	2.8	8.21–8.19

256 transients, an acquisition time of 2 s and a spectral width of 8113 Hz. Between each transient a delay of 5 s was set to allow for relaxation. Exponential line broadening of 0.5 Hz was applied prior to Fourier transformation. Spectra were referenced by using the methanol resonance. The total experiment time summed up to 30 min. To assess the method, limit of detection (LOD) and quantification (LOQ), linearity, precision, and recovery of the extraction process using the TLC-MS interface were investigated by triplicate measurements. Quantitative analysis was performed using the PULCON implementation in TOPSPIN 2.1.

Results and Discussion

Analysis of compound mixtures by NMR spectroscopy following a chromatographic separation step could be accomplished in different modes. For example the direct coupling of liquid chromatography (LC) to NMR spectroscopy is a very elegant form that has been commercially available since the 90s and LC-NMR/MS since 1999.[9,10] However this well established technology relies on dedicated costly hardware. A simple alternative technique to combine chromatography with NMR detection is the TLC-NMR approach using a commercial TLC-MS interface without any need for special NMR hardware.

To assess the ability of a routine 400 MHz NMR instrument to identify and quantify compounds separated by TLC, we analyzed several low molecular weight compounds (Table 2). First linearity of the NMR system, without involving a TLC step, was investigated in a concentration range between 10 µg/ml and 80 µg/ml. For all compounds R^2 values >0.997 could be achieved (Table 2). Limits of quantification were also assessed for individual compounds (results are displayed in Table 2) based on the NMR regions given in Table 1. These results agreed well with expected values for routine NMR measurements. In the next steps precision and recovery were inspected for the test compounds. For this, all samples were sprayed on TLC plates and developed with the method given in Table 1 and extracted by use of the TLC-MS interface from the TLC plate. Precision and recovery were determined by ^{1}H NMR spectroscopy. All values obtained were in excellent agreement with expectations (see Table 2). The amount of sample spotted onto the plate was in a concentration range commonly used for separations. These outcomes proved TLC-NMR to be a valuable tool for quantification and identification of TLC separated compounds (Fig. 1).

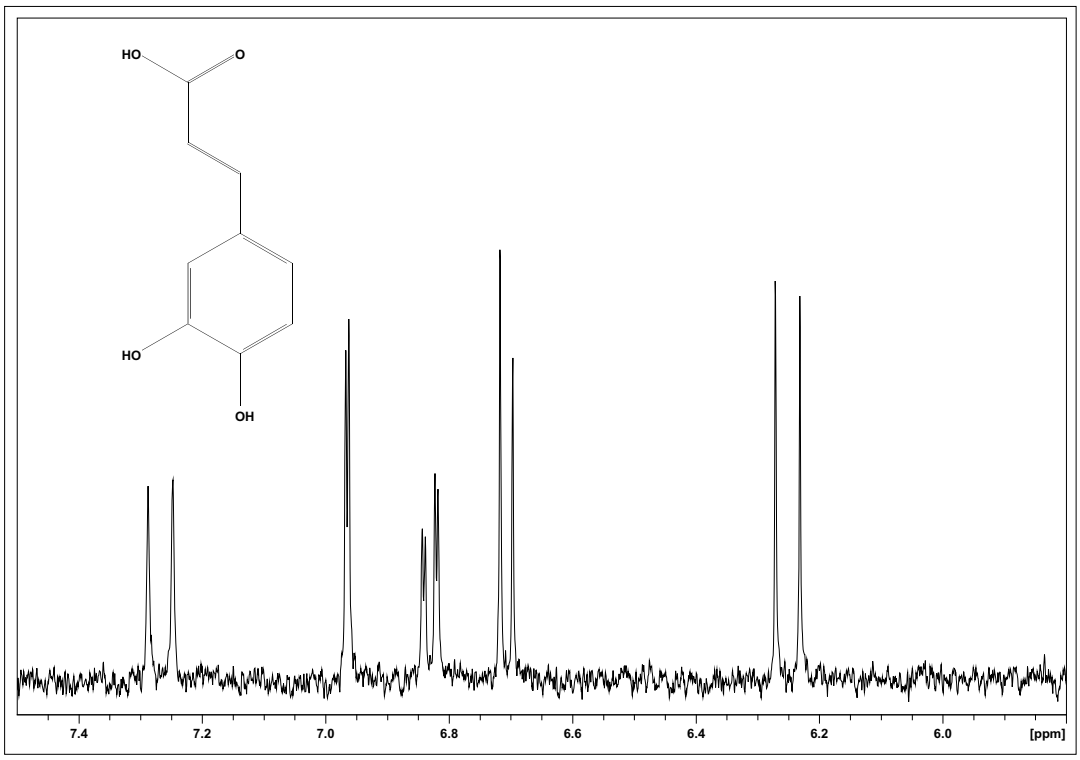

Fig. 1. Aromatic region of ^{1}H NMR Spectrum of 15.6 µg caffeic acid extracted from a TLC plate after development and measured at 400 MHz.

Table 2. Linearity, limit of quantification, precision and recovery determined by ^{1}H NMR spectroscopy for all tested compounds after development and extraction from TLC plates by use of the TLC-MS interface

Substance	NMR Linearity R^2 range: 10–80 µg/ml	LOQ (NMR) [µg]	Precision [%] RSD	Recovery [%]
Rutine	0.9976	6.9	3.9	101.8 ± 3.99
Caffeic acid	0.9978	7.3	1.5	103.4 ± 1.04
Chlorogenic acid	0.9991	10.1	3.1	100.5 ± 3.10
Amiodarone from Amiodarone Mepha 200 tablet	0.9998	8.5	2.7	100.5 ± 2.71

Conclusions

TLC–NMR is an interesting complementary method to the established TLC–MS combination. With no additional investment in additional NMR hardware the structure elucidation and quantification power of NMR spectroscopy can be exploited. Therefore TLC–NMR offers an interesting alternative to hyphenated NMR techniques like on-line LC-NMR spectroscopy. Quantification of API in formulation is possible after TLC separation on a routine 400 MHz NMR spectrometer within reasonable time for a concentration range down to 10 µg/ml. This matches well the commonly spotted sample amount in TLC separations although the NMR probe used in this work was not designed for highest proton NMR sensitivity. No prerequisites beyond the routine experimental steps in TLC–MS regarding selection, cleaning, or quality of TLC plates are necessary for TLC–NMR.

The ease of use of the TLC–MS interface to generate a sample for NMR quantification makes this technique an ideal tool for assessing the content of API in formulated drugs, determine degradation products or to quantify active components in plant extracts.

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[1] A. Schibli, E. Reich, *J. Planar Chromatogr. - Mod. TLC* **2005**, *18*, 34.
[2] H. Luftmann, M. Aranda, G. Morlock, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3772.
[3] S. Akoka, L. Barantin, M. Trierweiler, *Anal. Chem.* **1999**, *71*, 2554.
[4] G. F. Pauli, B. U. Jaki, D. C. Lankin, *J. Nat. Prod.* **2005**, *68*, 133.
[5] F. Malz, H. Jancke, *J. Pharm. Biomed. Anal.* **2005**, *38*, 813.
[6] U. Holzgrebe, *Prog. Nucl. Magn. Reson. Spectrosc.* **2010**, *57*, 229.
[7] G. Wider, L. Dreier, *J. Am. Chem. Soc.* **2006**, *128*, 2571.
[8] S. H. Smallcombe, S. L. Patt, P. A. Keifer, *J. Magn. Reson. A*, **1995**, *117*, 295.
[9] K. Albert, G. Schlotterbeck, U. Braumann, H. Händel, M. Spraul, G. Krack, *Angew. Chem.* **1995**, *107*, 1102.
[10] K. I. Burton, J. R. Everett, M. J. Newman, F. S. Pullen, D. S. Richards, A. G. Swanson, *J. Pharm. Biomed. Anal.* **1997**, *15*, 1903.