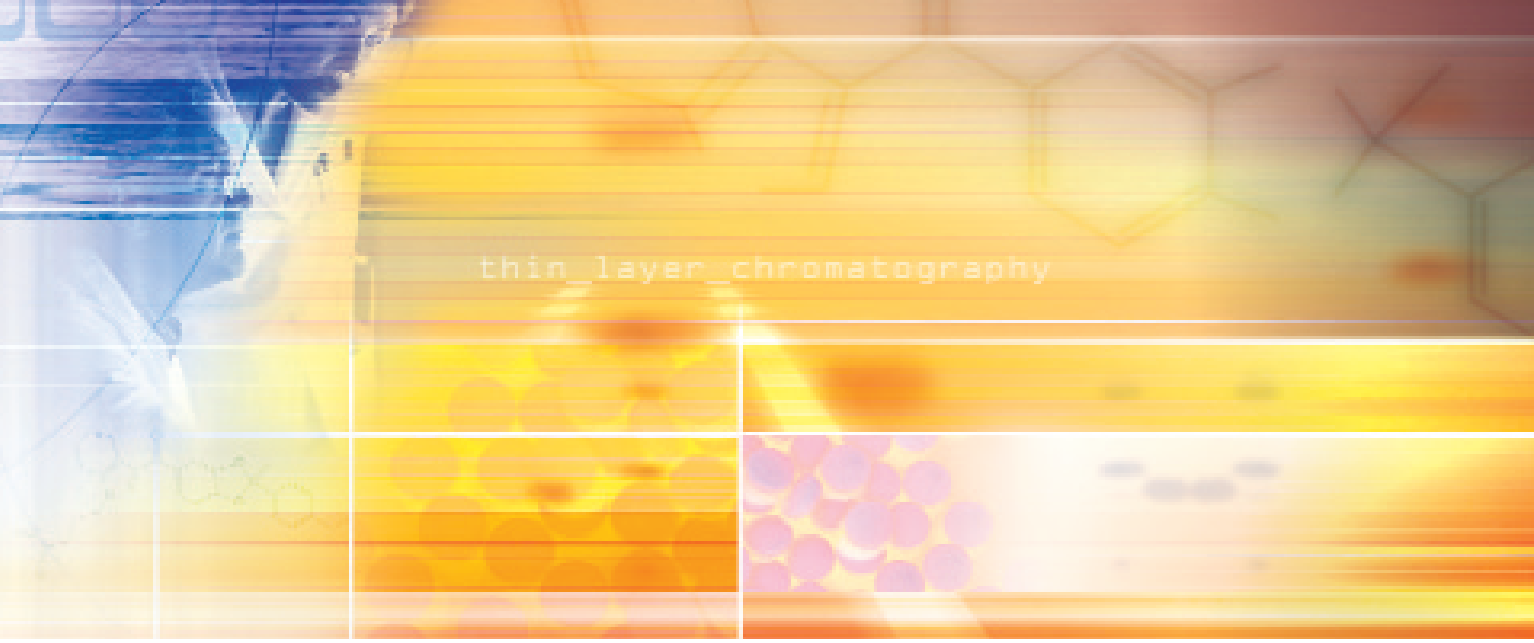

TLC–MALDI in Pharmaceutical Analysis

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Thin-layer chromatography (TLC) is a widely used technique for the rapid examination of compound purity; however, the amount of qualitative information obtained is limited to the retention factor (R_f) of compounds. Therefore, we have developed a technique for the direct determination of TLC plates by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). Data are presented from the qualitative analysis of a range of pharmaceutical compounds and related substances. Methods for the generation of quantitative data, by incorporation of an internal standard into the TLC development solvent, are described and the use of post-source decay (PSD)-MALDI experiments in conjunction with TLC–MALDI-MS for compound identification reported.

Introduction

Although replaced in many instances by high performance liquid chromatography (HPLC), thin-layer chromatography (TLC) is still important in the pharmaceutical industry for the analysis of compound purity. It is used either for simple "on the bench" experiments conducted by synthetic organic chemists or in manufacturing quality-control procedures. It can be used to analyse many samples simultaneously at low cost and with minimal equipment and operator training. However, if an unknown or unexpected spot appears, the only qualitative information that can be obtained from it is the retention factor (R_f) value. Consequently, it is current practice to reanalyse these samples by liquid chromatography–mass spectrometry (LC–MS). This may require HPLC method development, which can be costly and time consuming. The development of an on-line TLC–MS method would solve this issue.

Considerable effort has been made over the past few decades to combine TLC with (reviewed by Wilson)[1] fast atom bombardment (FAB), liquid secondary ion (LSI), laser two-step mass spectrometry (L2 MS), matrix-assisted laser desorption ionization (MALDI), surface-assisted laser desorption ionization (SALDI) and electrospray ionization (ESI) techniques. Of these the combination of TLC and MALDI offers the potential advantage of minimal analyte spreading, compared with TLC–FAB and TLC–LSI that both require the use of a liquid matrix (e.g., glycerol), and TLC–L2 MS that requires very complex and specialist instrumentation. Several groups, including ours, have identified the potential of MALDI-MS for the direct analysis of TLC plates. It has been applied to the analysis of a variety of polymers, including peptides and proteins,[2–5] nucleotides,[6] glycosphingolipids,[7] lipopolysaccharides[8] and styrene oligomers.[9–10] Low molecular weight compounds, such as dyes,[4,5,11] drugs[12] and pesticides[13] have also been investigated by TLC–MALDI-MS. The key to successful TLC–MALDI-MS analysis is the method used to prepare the TLC plates; that is, the method used for matrix application.[14] In this article we describe the TLC–MALDI technique we have developed for the analysis of pharmaceutically related compounds.[14–19] In our approach the matrix is electrosprayed onto the TLC plate; this has the major

benefit over alternative methodologies of maintaining chromatographic integrity such that chromatographic and mass spectral information may be obtained by scanning the TLC plate in a modified MALDI time-of-flight (TOF) mass spectrometer. In this brief overview, we aim to demonstrate the utility of the technique for the identification and quantification of pharmaceutical compounds and related substances.

Detection and Identification of Related Substances

Monitoring the presence of 'related substances' is crucial to the development of pharmaceutical compounds. The term 'related substance' describes compounds structurally similar to the drug, including synthetic impurities, degradation products and impurities arising from storage or manufacturing. The analysis of related substances is performed on both the bulk drug and the finished drug product. Sensitivity is crucial for this application because the allowed level of related substances in medicinal preparations is 0.1%.[20]

The applicability of our TLC–MALDI method for the detection of related substances at the 0.1% level is demonstrated here using the example of UK-137,457 (a compound synthesized by Pfizer Global R&D as part of a drug-development programme) and UK-124,912 (one of its related substances). The structures of both compounds are shown in Figure 1.

The methodology we have developed, which enables the detection of TLC spots on the basis of the relative molecular mass of the compounds under analysis, is summarized in Figure 2. After the TLC separation, a strip of the TLC plate (60 × 2 mm) is cut out and mounted onto a MALDI target using double-sided tape. The MALDI matrix is then electrosprayed onto the surface using a robotic fraction collector (BAI, Bensheim, Germany) modified to act as an electrospray deposition device. By applying a high voltage (~2.5 kV) to the spray capillary (Figure 2(b)) a very fine mist of droplets is formed; this creates a homogeneous matrix layer on the TLC plate surface (Figure 2(b) bottom) and prevents high levels of analyte spreading. The last step is the introduction of the prepared TLC sample into the MALDI-MS, to obtain chromatographic and mass spectral data (Figure 2(c)). We have found that the electrospray matrix deposition method produces both a stable mass spectrometric signal and maintains the chromatographic integrity of the analyte spots.

Figure 3 shows the results obtained from the direct TLC–MALDI analysis of UK-137,457, containing UK-124,912 at the 0.1% level. In these data, although the TLC spot from UK-124,912 is not observable in the UV data (Figure 3(a)), it can be seen clearly in the recorded mass chromatogram of *m/z* 412–413 (Figure 3(b)) at an R_f value of 0.63. The protonated molecule at *m/z* 412.8 in the mass spectrum (Figure 3(d)) corresponds to the expected value of UK-124,912. The matrix-related background can be seen in

Figure 1: Structures of (a) UK-137,457 ($C_{31}H_{31}NO_5$) and (b) UK-124,912 ($C_{27}H_{25}NO_3$). In both instances the formula weight (FW) quoted is calculated using the averaged isotopic atomic masses.

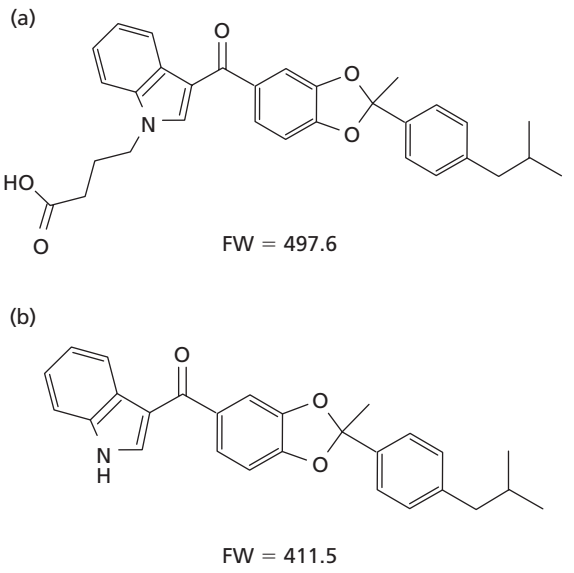
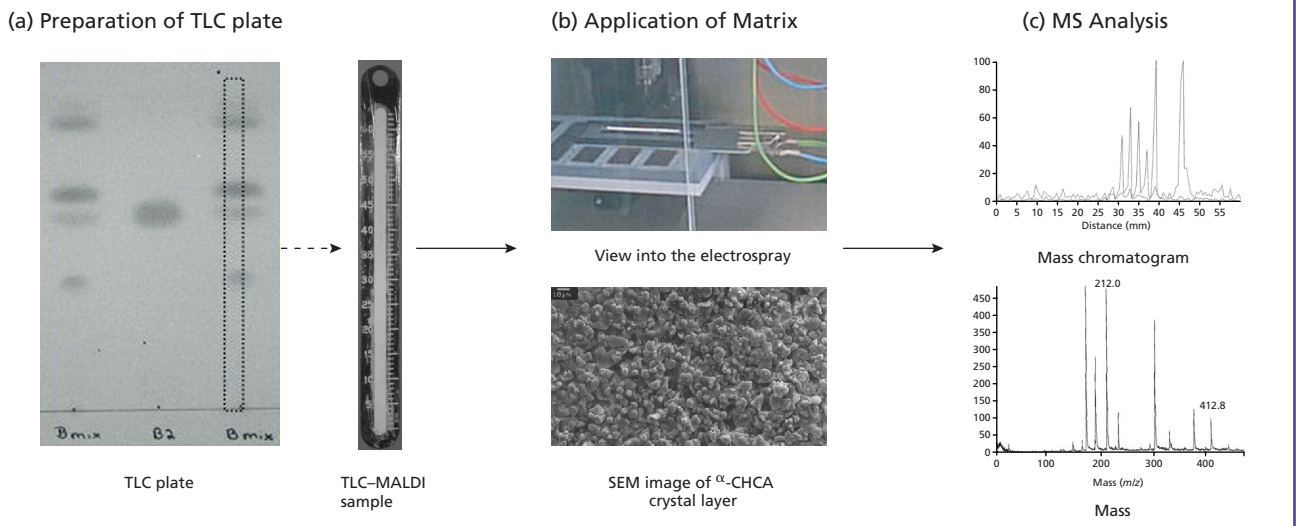


Figure 2: The sample-preparation steps in the TLC–MALDI experiments.



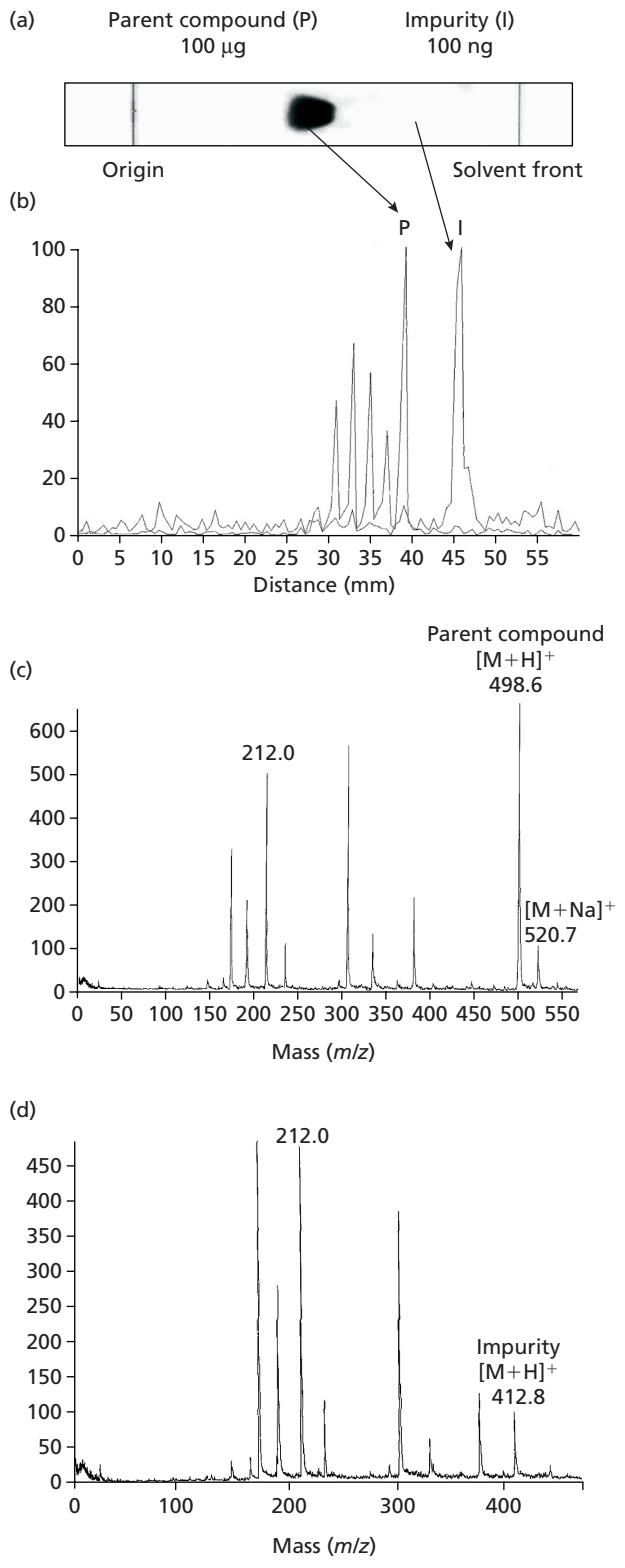


Figure 3: TLC–MALDI data obtained from the analysis of UK-137,457 containing UK-124,912 at the 0.1% level: (a) TLC separation, (b) overlaid mass chromatograms obtained by MS scan of the TLC plate, (c) TLC–MALDI spectrum of UK-137,457 and (d) TLC–MALDI spectrum of UK-124,912.

the low mass range (*m/z* 150–400) in both mass spectra (Figures 3(c) and (d)). The sodium adduct ion of the matrix (α-CHCA) at *m/z* 212 was used as an internal recalibration point during data acquisition to improve mass accuracy and mass resolution. Without internal recalibration the porosity of the silica gel layer leads to mass measurement inaccuracies and poor mass-spectral resolution in TLC–MALDI-TOF. Ions starting from different points on the surface can have a slight variation in their flight times. Therefore, a decrease in both parameters in the recorded spectra is still observed when a TOF analyser is employed to record data from an uneven surface, such as a TLC plate, even with internal recalibration. However, this technique reduces the degradation to acceptable levels.

The detection limit assessment of the related substance UK-124,912 revealed that mass chromatograms could be constructed from spots containing only 2.4 pmol of compound. Further improvements in sensitivity were achieved using a special Si 60 F_{254} high performance (HP)TLC–MALDI target (developed in collaboration with Merck KGaA, Darmstadt, Germany). A 100 µm thick layer of silica gel 60 sorbent was coated in the 60 × 2 mm recess of the MALDI target and small amounts of a polymer binder and fluorescence indicator were added. Using this special TLC–MALDI target mass spectra of 972 fmol UK-124,912 have been recorded.[21]

Post-source decay (PSD) is a technique used in MALDI-MS to generate structural information.[22] A large portion of the protonated molecules produced in MALDI-MS undergo extensive metastable decay in the TOF tube — this process is called post-source decay. The product ions formed by PSD continue to travel through the TOF tube with the same velocity, but with a range of kinetic energies because their mass has changed. Ions with a higher kinetic energy (i.e., heavier ions) penetrate deeper into a reflectron ("ion-mirror" part of the instrument) than lighter ions and hence mass-to-charge separation of the non-dissociated precursor ion and the product ions is achieved.

To facilitate the identification of an unknown TLC spot arising from a pharmaceutical compound or one of its related substances, TLC–PSD-MALDI may be performed directly on the separated TLC spots. In this instance a higher concentration of the studied compounds is required because the location of the highest analyte signals within the spot is obtained by scanning the whole TLC plate (first step), followed by PSD analysis at the "sweet spot" within the analyte spot (second step). Again, UK-137,457 and UK-124,912 were selected as examples. A 1% level of the related substance UK-124,912 was chosen (corresponding to a quantity of 1 µg). The protonated molecular species of UK-137,457 at *m/z* 498 and of UK-124,912 at *m/z* 412 were selected as the precursor ions. In this instance, to improve the mass accuracy obtained for spectra obtained from the TLC plate, the instrument used was recalibrated using the selected precursor ion at the TLC position from which the TLC–PSD-MALDI spectra were recorded.

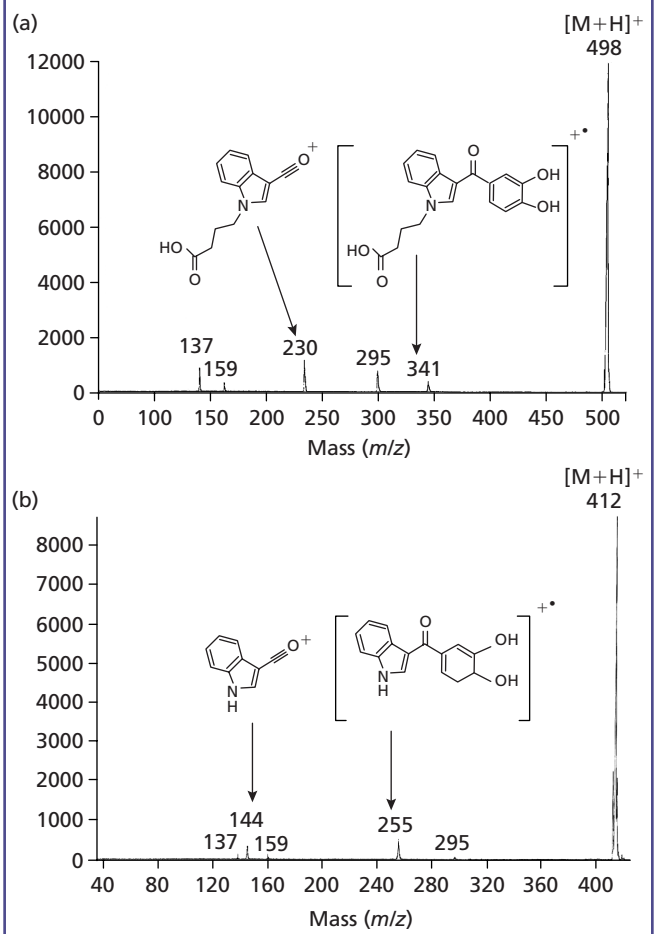
The TLC–PSD-MALDI spectra obtained are shown in Figures 4(a) and (b), respectively. The important product ions are those of *m/z* 230 and 341 for UK-137,457 and *m/z* 144 and 255 for UK-124,912. The difference in *m/z* value of 86 between corresponding ions is the formula weight of the side-chain group of the pyrrole ring of UK-137,457, allowing structural identification of UK-124,912.

We aim to further exploit the exciting potential for structural elucidation by this technique using a quadrupole–TOF hybrid mass spectrometer (Q-TOF) for full tandem MS experiments. As well as the improved precursor ion selection possible on such an instrument, preliminary experiments have shown that the requirement for on-line recalibration of data is removed. The first quadrupole stage of the instrument completely detaches the TOF mass analyser from the ion formation process, eliminating effects arising from the uneven TLC plate surface.[21]

Quantitative Aspects

The quantitative determination of pharmaceutical compounds by TLC–MS has been the subject of several reports, such as an off-line TLC–SPE–APCI-MS determination of caffeine[23] and several on-line TLC–MS determinations, including the quantification of pyridostigmine[24] and nicerogline[25] by TLC–LSI MS, the diuretic amiloride by TLC–FAB MS,[26] imipramine by TLC–L2 MS[27] and cocaine by TLC–MALDI-MS.[12] In the majority of these quantification experiments a chemical or stable isotope analogue was incorporated into the experiment as internal standard to enable quantification to be performed directly on the separated TLC spots.

Figure 4: TLC–PSD-MALDI spectra of (a) UK-137,457 (100 µg) and (b) UK-124,912 (1 µg) using α-CHCA as matrix. Adapted from reference 18.



The non-steroidal anti-inflammatory drug (NSAID) piroxicam or 4-hydroxy-2-methyl-N-(2-pyridyl)-H-1,2-benzothiazine-3-carboxamide-1,1-di-oxide is widely used in the treatment of rheumatological disorders. Several analytical techniques have been used for the determination of piroxicam including TLC, capillary electrophoresis (CE), spectrofluorimetry, derivative spectrometry and HPLC. The main advantages arising from the development of a TLC–MALDI-MS method would be its simplicity, sensitivity and specificity. Hence, our aim was to develop and validate a quantitative TLC–MALDI-MS method for the analysis of piroxicam. Tenoxicam, a structural analogue of piroxicam, was used as internal standard to compensate for MALDI-MS signal deviations and variations in the extraction efficiency of piroxicam from the TLC plate. To achieve this, the internal standard must be located at the same position as the analyte. Preliminary data, obtained by spotting mixtures containing the internal standard and the analyte, on silica gel 60 F_{254} TLC plates proved that quantification of piroxicam with the chosen internal standard was possible. As the internal standard did not have the same R_f value as the analyte in the TLC analysis, in contrast to the quantitative TLC–MS methods described in the literature, a suitable method of incorporating the internal standard into the experiment had to be established. The following approaches were tested:

- development of the TLC plate in a mobile phase to which the internal standard was added
- electrospraying of a solution of tenoxicam onto the plate
- electrospraying a mixture of tenoxicam and matrix (α-CHCA) onto the plate.

The best precision for the standard calibration curve was obtained by incorporating the internal standard in the mobile phase (7–28% RSD, n = 4), as shown in Figure 5. The relatively limited linear range (400–800 ng) is believed to be caused by ion suppression effects that are well documented for MALDI.[28] The points of the calibration curve were obtained by dividing the integrated area of the piroxicam signals by the integrated area of the tenoxicam signals at the same position on

Figure 5: Standard calibration curve obtained from the TLC–MALDI analysis of piroxicam. The plotted data points of the calibration curve are calculated from the corresponding mass chromatograms and are the arithmetic means of four experiments. Error bars indicate the standard deviations. Adapted from reference 19.

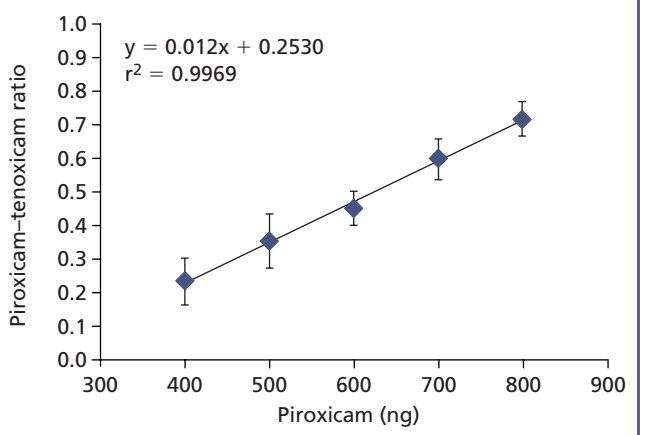


Table 1: Precision and accuracy results of the TLC–MALDI determination of piroxicam. Adapted from reference 19.

Piroxicam (ng)	Detected (ng)*	RSD (%)	Accuracy (%)
450	457 ± 41	8.9	1.5
600	586 ± 20	3.5	2.3
750	745 ± 10	1.3	0.7

*Mean of 5 determinations ± standard deviation.

the TLC plate in the recorded mass spectra.

The method was assessed using standard criteria:[29] accuracy, precision, specificity, limit of detection (LOD), limit of quantification (LOQ), linearity and range of the measurements. (The robustness could not be determined because the methodology is currently unique to our laboratory). The LOD for piroxicam was calculated as 39 ng and the LOQ as 131 ng. These values are similar to the ones described for the quantitative HPTLC determination of piroxicam (LOD = 40 ng, LOQ = 150 ng).[30] Table 1 summarizes the results for the method validation. Good accuracy and precision were achieved by this approach. The simplicity of predeveloping the TLC plate with an appropriate internal standard to perform quantification in TLC–MALDI-MS makes this technique particularly attractive.

Conclusions

Several of the issues that have arisen in the development of TLC–MALDI-MS methods for the successful analysis of pharmaceuticals have been addressed in this article. The first concerns a method for the deposition of the MALDI matrix onto the TLC plate. This electrospray deposition method was found to be superior to other methods studied and was successfully applied to a range of compounds presented. To aid in structural evaluation of the analysed compounds, it has been demonstrated that PSD analysis can be performed directly on the TLC spots. The generation of quantitative data using a structural analogue as internal standard and incorporation into the mobile phase has also been demonstrated.

The next step in the development of TLC–MALDI-MS in pharmaceutical analysis would be its more widespread use in industry. For this, further developments are necessary that enhance sensitivity, mass resolution and reproducibility. The availability of commercial instruments that allow the scanning of whole TLC plates rapidly with data-imaging software would facilitate this development. The combination of TLC with MALDI is not only applicable to the analysis of pharmaceuticals and we hope that this brief overview might inspire other researchers to investigate its applicability in other areas.

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References

1. I.D. Wilson, *J. Chromatogr. A,* **856**, 429 (1999).
2. A.I. Gusev et al., *Anal. Chem.,* **67**, 1805 (1995).
3. A.I. Gusev et al., *Anal. Chem.,* **67**, 4565 (1995).
4. J.T. Mehl, A.I. Gusev and D.M. Hercules, *Chromatogr.,* **46**, 358 (1997).
5. J.T. Mehl and D.M. Hercules, *Anal. Chem.,* **72**, 68 (2000).
6. D.T. Isbell et al., Fresenius *J. Anal. Chem.,* **365**, 625 (1999).
7. J. Guittard, X.L. Hronowski and C.E. Costello, *Rapid Commun. Mass Spectrom.,* **13,** 1838 (1999).
8. H. Therisod, V. Labas and M. Caroff, *Anal. Chem.,* **73**, 3804 (2001).
9. K. Matsumoto et al., *J. Mass Spectrom. Soc. Jpn,* **47,** 274 (1999).
10. K. Matsumoto et al., *J. Mass Spectrom. Soc. Jpn,* **49**, 49 (2001).
11. A.W.T. Bristow and C.S. Creaser, *Rapid Commun. Mass Spectrom.,* **9**, 1465 (1995).
12. A.J. Nicola, A.I. Gusev and D.M. Hercules, *Appl. Spectroscopy,* **50**, 1479 (1996).
13. R.L. Vermillion-Salsburg et al., *Intern. J. Environ. Anal. Chem.,* **73**, 179 (1999).
14. S. Mowthorpe et al., *Rapid Commun. Mass Spectrom.,* **13**, 264 (1999).
15. A. Crecelius et al., *J. Planar Chromatogr.,* **13**, 76 (2000).
16. A. Crecelius et al., *J. Chromatogr., A.* **958**, 249 (2002).
17. A. Crecelius et al., in "Advances in Mass Spectrometry Volume 15," E. Gelpi, J. Wiley and Sons, Ed., (Chichester, West Sussex, UK, 2001), 695–696.
18. A. Crecelius et al., *J. Chromatogr. Sci.,* **40**, 614 (2002).
19. A. Crecelius et al., *J. Pharm. Biomed. Anal.,* (Submitted).
20. FDA Guidance, Q3B Impurities in New Drug Products, http://www.fda.gov/cder/guidance/1317fnl.htm
21. A. Crecelius, PhD Thesis "Thin Layer Chromatography–Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry of Pharmaceutical Compounds," Sheffield Hallam University 2002.
22. B. Spengler, *J. Mass Spectrom.,* **32**, 1019 (1997).
23. M. Prosek et al., *J. Planar Chromatogr.,* **13**, 452 (2000).
24. M.S. Stanley, K.L. Busch and A. Vincze, *J. Planar Chromatogr.,* **1**, 76 (1988).
25. K. Banno, M. Matsuoka and R. Takahashi, *Chromatogr.,* **32**, 179 (1991).
26. S.M. Brown and K.L. Busch, *J. Planar Chromatogr.,* **4**, 189 (1991).
27. L. Ling and D.M. Lubman, *Anal. Chem.,* **61**, 1911 (1989).
28. W.R. Wilkinson et al., Fresenius *J. Anal. Chem.,* **357**, 241 (1997).
29. FDA Draft Guidance for Industry: Analytical Procedures and Methods Validation (August 2000), http://www.fda.gov/cber/gdlns/methval.pdf
30. S.P. Puthli and P.R. Vavia, *J. Pharm. Biomed. Anal.,* **22**, 673 (2000).

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