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Quantitative determination of Piroxicam by TLC–MALDI TOF MS

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Abstract

A quantitative thin-layer chromatography (TLC)–matrix-assisted laser desorption (MALDI) TOF mass spectrometry (MS) method for the determination of Piroxicam has been developed. Following preliminary experiments three different approaches to the incorporation of the internal standard (Tenoxicam) into the TLC plates were investigated. These were: (a) adding the internal standard to the mobile phase and pre-developing the plate, (b) coating the plate with internal standard by electrospraying prior to matrix application and finally, (c) mixing the internal standard into the matrix solution and electrospraying both. The most successful method was that where the internal standard was pre-developed over the plate. For this method linearity was observed over the range between 400 and 800 ng of Piroxicam. The precision was found to be in the range of 1–9% R.S.D. from the average detected value ($n = 5$), dependent on the amount of analyte on the TLC plate. The proposed method was accurate with ±2% deviation from the known amount of Piroxicam in the sample spot.

Keywords: Quantitative analysis; Piroxicam; Tenoxicam; TLC; Matrix assisted laser desorption ionisation; Mass spectrometry

1. Introduction

Piroxicam or 4-hydroxy-2-methyl-*N*-(2-pyridyl)-H-1,2-benzothiazine-3-carboxamide-1,1-di-oxide is a non-steroidal anti-inflammatory drug (NSAID) [1] widely used in the treatment of rheumatological disorders [2]. Several analytical methods have been described for the determination of Piroxicam, including thin-layer chromatography (TLC) [3–6], capillary electrophoresis (CE) [7], spectrofluorometry [8], derivative spectrometry [9–11] and high performance liquid chromatography (HPLC) [12–15].

In recent years there has been an increased interest in the use of matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS) for the direct analysis of TLC plates [16]. There are several issues, which have to be addressed for successful TLC–MALDI coupling. The first is concerned with the method used for the deposition of the MALDI matrix onto the TLC plate. This matrix facilitates the ionisation of the analyte molecules separated on the TLC plate. The application of the matrix directly

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onto the TLC plate has to be done in such a manner that analyte spreading across the silica gel layer is avoided. This maintains the chromatographic integrity of the analyte spots. The second issue is the requirement for efficient extraction of the analyte from the interior of the silica gel layer to increase the sensitivity. The porosity of the silica gel layer, can also lead to problems, ions starting from different points in the surface can have a slight variation in their flight times and hence lead to a decrease in the mass resolution of spectra recorded. Finally for the analysis of low molecular weight compounds, such as pharmaceuticals, strong matrix peaks can interfere with the analyte signal.

Several approaches for the application of the matrix for TLC–MALDI coupling have been compared including a recently developed electrospray matrix deposition method [17]. This electrospray method was found to be superior to the other techniques studied. It produced a stable signal, minimised analyte spreading and hence allowed the scanning of a TLC plate to obtain chromatographic as well as mass spectral data. The use of an extraction solvent prior the matrix application has been shown to enhance sensitivity [18]. Mass measurement inaccuracies observed in TLC–MALDI TOF MS can be corrected by internal recalibration on selected matrix ions during the scanning of a TLC plate [18]. The identification of compound spots [19] is aided by the application of post-source decay (PSD) techniques to MALDI–TLC MS analysis. The minimisation of matrix interferences has been addressed by applying suspensions of particles of different materials and sizes (Co-UFP, TiN, TiO_2, graphite and silicon) onto eluted TLC plates [20]. To date little work has been conducted on the quantitative determination of pharmaceuticals by TLC–MS. There appear no reports on the application of TLC–MALDI TOF MS on the determination of the pharmaceutical Piroxicam. Caffeine has been quantified by off-line TLC–SPE–APCI–MS [21]. On-line TLC–MS techniques, which have the advantage of obtaining mass spectral as well as chromatographic information with respect to the TLC spots, have been used for the analysis of nicerogline by TLC–SIMS [22], and the analysis of cocaine by TLC–MALDI [23]. In both approaches a chemical or stable isotope analogue was used as internal standard to enable quantification to be carried out directly on the separated TLC spots.

We report the development and validation of a quantitative TLC–MALDI TOF MS method for the analysis of Piroxicam. Tenoxicam, a structural analogue of Piroxicam, was used as internal standard to compensate MALDI MS signal deviations and variations in the extraction efficiency of Piroxicam from the TLC plate. To regulate the analyte signals, recorded directly from the TLC plate, the internal standard has to be located at the same positions as the analyte. Preliminary data obtained by spotting mixtures containing the internal standard Tenoxicam and the analyte Piroxicam on silica gel 60 F_{254} TLC plates showed that quantification of Piroxicam with the chosen internal standard was possible. The critical step was to find a suitable approach of incorporating the internal standard on the TLC plate, so that the development of the TLC plate was possible. Since the internal standard did not show the same R_f value as the analyte in the TLC analysis, the following approaches were tested: development of the TLC plate in the mobile phase to which the internal standard was added, electrospraying of a solution of Tenoxicam on to the TLC plate and electrospraying a mixture of Tenoxicam with the MALDI matrix.

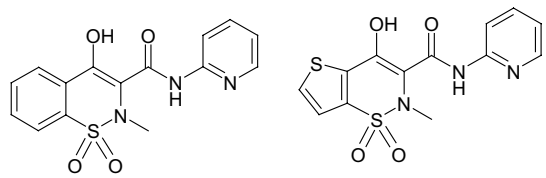
2. Experimental

2.1. Materials

Piroxicam (PX) and Tenoxicam (TX) were obtained from Sigma-Aldrich (Dorset, UK). The chemical structures are shown in Fig. 1.

2.2. Instrumentation and conditions

For the application of the MALDI matrix on the silica gel surface of the TLC plate, an in-house modified commercial robotic *x*–*y*–*z*-axis motion system



Piroxicam (PX, MW 331) Tenoxicam (TX, MW 337)

Fig. 1. Chemical structures of Oxicam derivatives.

(PROBOT, BAI, Germany) was used. Modifications to the instrument are described elsewhere [19,20].

Mass spectra were recorded directly from TLC strips with a modified linear Laser TOF 1500 mass spectrometer (SAI, UK), equipped with a nitrogen laser ($\lambda = 337$ nm). The modifications to the instrument and its software have been described previously by this group [18]. Experiments were carried out using a 10 kV extraction voltage. Only the positive ion mode was used and the mass spectra acquired from the TLC surface were the results of the cumulative acquisition of 16 shots. The TLC strips were scanned over a distance of 50 or 55 mm and mass spectra were recorded every 0.5 mm. A data set of 100 or 110 mass spectra was obtained for each TLC strip.

2.3. Quantification methods

The section is divided into four sub-sections 1, 2, 3 and 4, which describe the different approaches for the incorporation of the internal standard Tenoxicam into the TLC plate. *Method 1*: Describes the preliminary experiments where the internal standard was simply mixed with the analyte solution the resulting mixture then spotted onto the TLC plate. *Method 2*: Pre-development of the TLC plate in the mobile phase to which the internal standard is added, followed by development of the analyte in the same mobile phase. *Method 3*: Electrospraying of the internal standard prior matrix application. *Method 4*: Mixing of the internal standard with the matrix solution and electrospraying of the resulting solution.

2.3.1. Calibration standards

Method 1: Primary stock solutions of the analyte PX (1 mg/ml) and the internal standard TX (2 mg/ml) were prepared in hydrochloric acid (2.0 M), respectively. Five standard solutions of PX (0.4, 0.5, 0.6, 0.7 and 0.8 mg/ml) with a constant concentration of TX (0.4 mg/ml) were obtained by combining aliquots of both primary solutions and diluting with HPLC-grade methanol.

Method 2: Primary stock solution of the analyte PX (1 mg/ml) was prepared in HPLC-grade dichloromethane and five standard solutions (0.4, 0.5, 0.6, 0.7 and 0.8 mg/ml) were obtained by subsequent dilution of the primary stock solution.

Methods 3 and 4: Five standard solutions of PX (0.4, 0.5, 0.6, 0.7 and 0.8 mg/ml) were obtained by dilution the primary stock solution of PX of method 1 with HPLC-grade methanol.

2.3.2. Sample preparation

Method 1: Aliquots of standard solutions (1 μl) were applied to an aluminium-backed TLC plate coated with a 0.2 mm layer of silica gel 60 F254 (Merck, Germany) using disposable spotting pipettes (Camag, Switzerland). A distance of 10 mm between the analyte spots was chosen to keep them separate, in order to be able to detect 5 spots in a single experiment. After UV detection ($\lambda = 254$ nm) of the analyte spots, a strip of the TLC plate (2 mm × 50 mm) was cut out and attached to a modified MALDI target using double sided tape. The organic matrix α-cyano-4-hydroxy cinnamic acid (α-CHCA) (Sigma-Aldrich, Dorset, UK) was dissolved in HPLC-grade methanol containing 0.1% TFA. A solution of 20 mg/ml of α-CHCA was electrosprayed on to the silica gel TLC strip and after 30 min. drying at room temperature, the TLC sample was subjected to MALDI analysis.

Method 2: The TLC plate described previously was pre-developed in 25.0 ml chloroform–methanol (9:1 (v/v)), in which TX was dissolved to a mass concentration of 0.4 mg/ml. The chamber (dimensions 12.1 cm × 10.8 cm × 8.3 cm, Sigma-Aldrich, Poole, UK) was lined with filter paper and saturated for 30 min. The TLC plate was developed for 17 h in the mobile phase to obtain a homogeneous surface coverage of TX. After air drying, 1 μl aliquots of standard solutions were applied to the pre-developed TLC plate and the plate was developed again in the same mobile phase to a distance of 7.0 cm (The mobile phase was freshly prepared and saturated for 1 h prior use). The developed TLC plate was air dried and electrosprayed with α-CHCA prior MALDI analysis, as described for method 1.

Method 3: Aliquots of standard solutions (1 μl) were applied to the TLC plate as described in method 1. The plate was developed as described in method 2. Following this a 0.25 mg/ml solution of TX dissolved in acetone was electrosprayed onto the TLC plate and finally the matrix solution was coated onto the plate by electrospraying.

Method 4: A 0.1 mg/ml solution of TX dissolved in dichloromethane was mixed into the matrix solution

in the volume ratio 1:1 and electrosprayed on the analyte spots. Chromatographic conditions as described in Methods 1 and 2.

2.3.3. Calibration curves

Standard curves were constructed for samples spots containing 400–800 ng of PX for each method. Peak responses of PX and TX were measured in each of the mass spectra recorded. From these data mass chromatograms of PX and TX were generated for each sample (Fig. 3.), and the Piroxicam-to-Tenoxicam ratio for each single spot on the TLC strip was calculated using the software package Origin (OriginLab Corporation). For each spot, represented in the mass chromatograms as a peak, the Piroxicam-to-Tenoxicam ratio was obtained by dividing the integrated area of the PX signals by the integrated area of the TX signals at the same positions on the TLC plate. The Piroxicam-to-Tenoxicam ratios obtained by this methodology, were plotted versus the corresponding PX quantity. The calibration curve was determined by least-square linear regression analysis.

2.3.4. Method validation

Methods 1 and 2 were assessed by the following criteria [24]: accuracy, precision, specificity, limit of detection, limit of quantification, linearity and range of measurement. The robustness could not be determined since the methodology is currently unique to our laboratory.

2.3.4.1. Accuracy and precision. To evaluate the accuracy and precision of the proposed methods three quantities within the previously determined linear range (450, 600 and 750 ng PX) were selected and analysed. From each quantity ten determinations (method 1) or 5 determinations (method 2) were performed. The accuracy of the method was defined as the amount of Piroxicam determined by TLC–MALDI MS expressed as a percentage of the "true" amount of PX in the sample spot. To be acceptable, measures should be within ±15% at all concentrations [25]. The precision of the method was expressed as the relative standard deviation (R.S.D.) of replicate analyses, carried out within one working day. To be acceptable, measures should be lower than ±15% at all concentrations [25].

2.3.4.2. Specificity. Six blank TLC plates were tested for the presence of interfering peaks arising from the silica gel layer and the matrix.

2.3.4.3. Limit of detection and quantification. The limit of detection (LOD) was defined as the PX amount resulting in a peak area of three times the noise level. The limit of quantification (LOQ) was defined as the PX amount resulting in a peak area of ten times the noise level. The LOD and LOQ value were estimated by extrapolation from the constructed calibration curves [26].

2.3.4.4. Range. The range was determined by selecting three amounts of PX (a lower, middle and upper amount) and demonstrating that the analyses could be obtained with a suitable level of precision, accuracy and linearity.

3. Results and discussion

3.1. Matrix selection

The suitability of the MALDI matrices α-cyano-4-hydroxy cinnamic acid (α-CHCA), 2,5-dihydroxybenzoic acid (DHB), 3-hydroxypicolinic acid (3-HPA), 2(-4-hydroxyphenylazo)benzoic acid (HABA) for the quantitative analysis of PX by TLC–MALDI TOF MS was studied. The selection of the matrix was based on examination of their MALDI-MS behaviour on stainless steel substrates, as previously described [17]. The matrix α-CHCA was found to give the best results based on the criteria described in reference [17], i.e. absence of interfering peaks in the spectral region of interest and reproducible analyte signal intensity from laser spot to spot over the target (good crystal homogeneity).

3.2. Internal standard selection

For quantitative MALDI analysis an internal standard is required to improve shot-to-shot and sample-to-sample reproducibility. The selection of an appropriate internal standard is critical for the success of such an analysis [27]. An isotopically labelled analogue is an ideal internal standard [28] in mass spectrometry, however these are seldom available

and structural analogues are often employed e.g. for the quantification of biomolecules [29] or small molecules [30] by MALDI–MS.

The selection of a suitable internal standard for quantification using TLC–MALDI was based on two important properties of the internal standard. Firstly it needs to be chemically similar to the analyte so that similar extraction efficiencies out of the silica gel phase are achieved. Secondly, for these experiments, a reasonable, mass separation between analyte and internal standard signal was required, since the linear TOF instrument employed in these investigations has a mass resolution of only ∼425 (FWHM). For these reasons Tenoxicam (TX) a structural analogue of PX was chosen. TX is also an *N*-heterocyclic carboxamide derivative of benzothiazine-1,2-dioxide and has been previously used as internal standard for the quantitative HPLC analysis of PX [15]. The mass difference of 6 mass units gave adequate resolution of the protonated molecules (Fig. 2.).

3.3. TLC–MALDI analysis

3.3.1. Method 1

These preliminary experiments involving spotting mixtures containing 400–800 ng of PX and the same amount of TX (400 ng) on untreated silica gel TLC plates were carried out to prove that quantification of PX with TX as internal standard is possible by TLC–MALDI TOF MS and to show that the method could be validated. The mass chromatograms obtained for the analyte PX (*m*/*z* range: 331–333) and the internal standard TX (*m*/*z* range: 337–339) using this approach are presented in Fig. 3A. PX showed a higher signal response compared to TX, and appears to give a better MALDI response. PX was increased in the spots from the left to the right side in Fig. 3A, a steady increase in the recorded signal intensities was not obtained. Moreover, all five spots contained the same amount of TX, but the signal responses varied greatly. These variations are caused by local

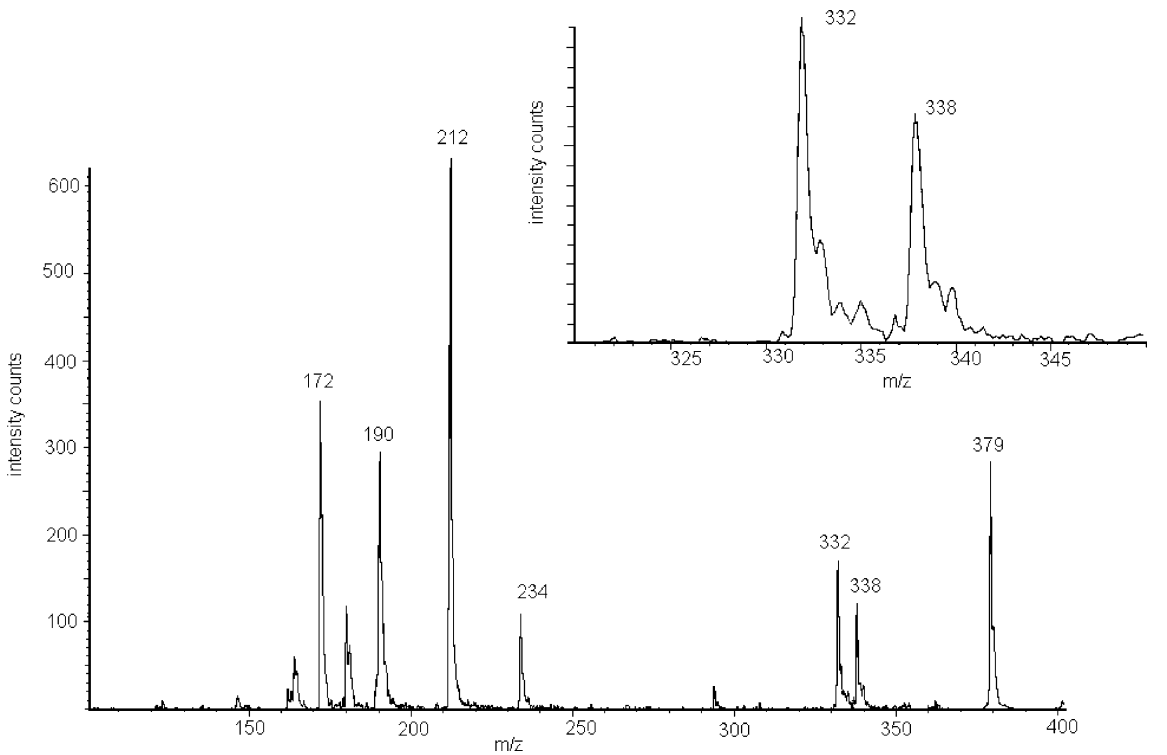


Fig. 2. TLC–MALDI mass spectrum of 600 ng of Piroxicam (method 2). The internal standard Tenoxicam (0.4 mg/ml in mobile phase) was pre-developed on the TLC plate. The insert shows the region around Piroxicam (*m*/*z* 332) and Tenoxicam (*m*/*z* 338).

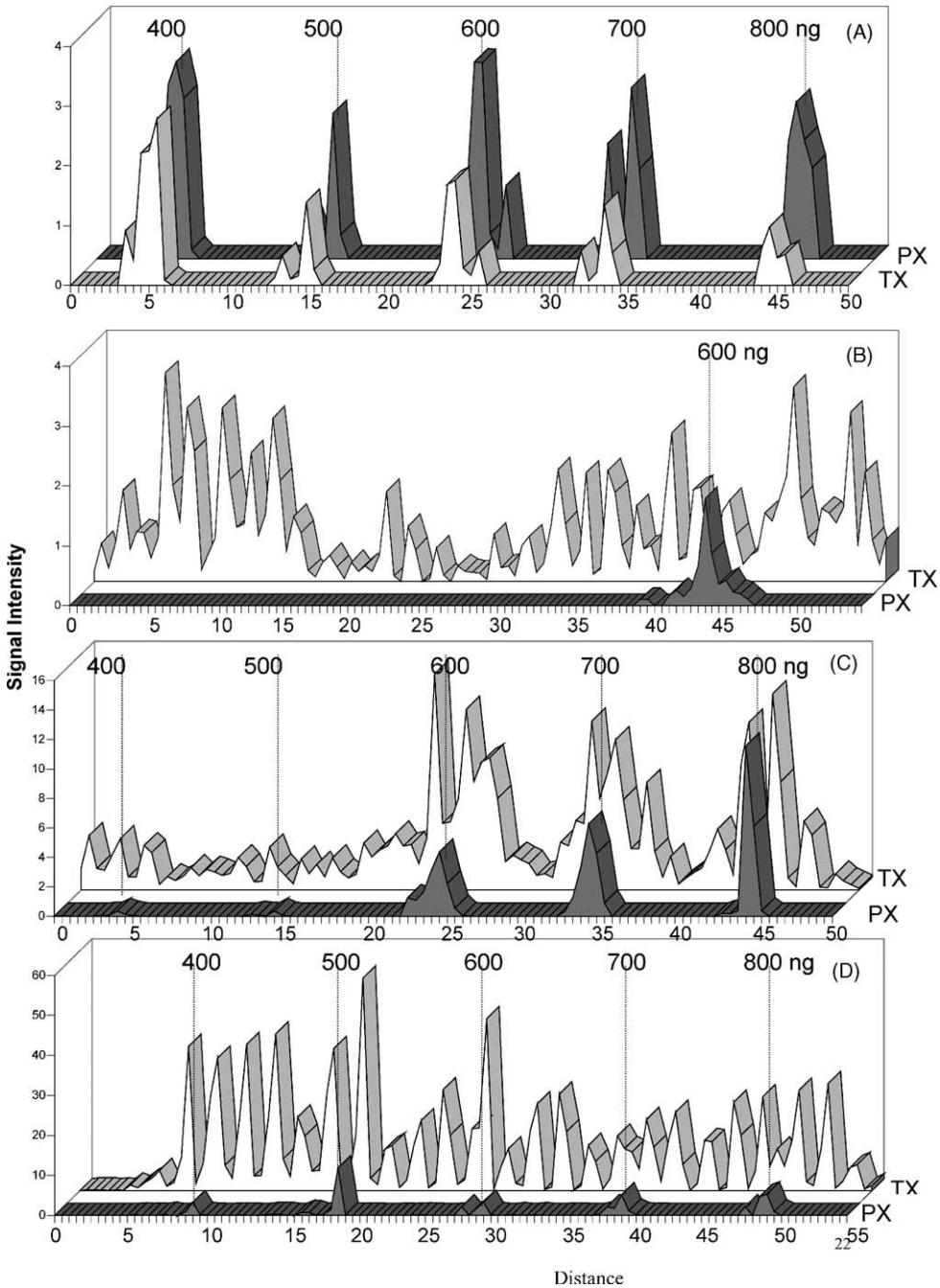


Fig. 3. Mass chromatograms obtained by different approaches of incorporating the internal standard: (A) method 1; (B) method 2; (C) method 3 and (D) method 4 (the methods are explained in Section 2). In method 1, 3 and 4 the quantities of Piroxicam (400–800 ng) were spotted on the TLC plate.

concentration changes of the compounds in the crystal structure [27]. The reason for these variations can be explained as follows. The solvent in which the matrix is dissolved acts as an extraction solvent of the compounds spotted on the TLC plate (analyte and internal standard). The extraction efficiency of the solvent throughout the scanned TLC strip varies from position to position and causes these variations. This shows how important the use of an internal standard is to normalise MALDI signals from a TLC plate when attempting quantification.

An effect where the signal from a less concentrated component is suppressed by that from a more concentrated one has been described for MALDI-MS [27,29]. In our TLC–MALDI experiments this effect lead to the limited linear range (400–800 ng) obtained in the constructed calibration curve. Suppression of the analyte occurred when a higher concentration than 0.4 mg/ml of the internal standard was used. Similarly the analyte concentration can also cause suppression of the internal standard signal, e.g. a quantity of 3 μg of PX suppressed 0.8 μg of TX.

3.3.2. Method 2

The mass chromatograms obtained by method 2 are shown in Fig. 3B. Since TX has a smaller R_f value ($R_f = 0.37$) than PX ($R_f = 0.63$), pre-development of the silica gel TLC plate with the internal standard was necessary to achieve the same location for analyte and internal standard. The variation of the signal responses of the internal standard was 90% R.S.D. from the averaged value throughout the scanned TLC strip. However, only the surface coverage at the location of the PX spot was of interest; and there the variation tracked the variation of the analyte.

Even with the matrix electrospray deposition method, which we have previously shown to produce a homogenous layer, compared to other methods of matrix application studied [17], variation of the matrix signals throughout the scanned TLC plate is observed. The variation of the signals of the internal standard tracks this as indicated by plotting the signal intensities of the sodium adduct of α-CHCA (*m/z* 212) and the protonated molecular signal of TX against the position on the TLC plate (data not shown).

The resulting calibration curve obtained by method 2 shows a four times smaller slope value ($b = 0.0012$) in comparison to calibration curves constructed from the preliminary data of method 1($b = 0.0047$). The full equations for the calibration curves in these cases were method 1, $y = 0.0047x - 0.6633 (R^2 = 0.9992)$ and method 2 $y = 0.0012x - 0.2530 (R^2 = 0.9969)$.

Fig. 2 shows the MALDI mass spectrum recorded at 45 mm of the scanned TLC plate, with the $[M+H]^+$ ion of PX clearly visible at *m/z* 332 and that of TX at *m/z* 338. Three matrix peaks of α-CHCA: $[M\text{-}H_2O + H]^+$ at *m/z* 172, $[M + H]^+$ at *m/z* 190 and $[2M + H]^+$ at *m/z* 379 were used as "Lock Masses" to overcome the degradation in mass measurement accuracy and mass resolution, observed when analysing TLC plates by TLC–MALDI MS, as previously reported [17,18].

3.3.3. Method 3

When the internal standard is electrosprayed on the analyte spots, care has to be taken to minimise planar spreading of the analyte. This can be achieved by using a non-polar solvent, in which the analyte is only sparingly soluble but which has reasonable solvating properties for the internal standard. This criterion is difficult to achieve due to the chemical similarity of analyte and internal standard. It was found that acetone is a suitable candidate, since its polarity index is 5.4 [31] and the solubility of TX is 2 mg/ml in it [32]. The concentration of TX in acetone was reduced from 0.4 to 0.25 mg/ml, since analyte suppression occurred at higher concentrations.

In the mass chromatograms shown in Fig. 3C, the increase in the internal standard response at the analyte spots containing 600, 700 and 800 ng of PX is significant. A possible explanation could be as follows. The internal standard molecules are located at a layer above the analyte molecules, and when the solvent of the matrix solution is extracting the analyte molecules from the inner part of the silica gel layer to the surface, they have to pass through the layer of the internal standard molecules. An increase in the concentration of the analyte molecules possibly causes an increase of internal standard molecules desorbed on the TLC plate surface. The variation of the internal standard signals along the TLC strip varied around 102% from the average value. The calibration curve exhibited an R.S.D. of 50% for the lowest amount of PX (400 ng) spotted on the TLC plate and one of 38% for the highest amount (800 ng) which discounts this method for validation. The full equation for the calibration curve in this case was $y = 0.0009x - 0.3173 (R^2 = 0.9969)$.

Table 1
Precision and accuracy of the TLC–MALDI methods for the determination of Piroxicam

Piroxicam (ng)	Method 1			Method 2		
	Detected (ng)[a]	R.S.D. (%)	Accuracy (%)	Detected (ng)[b]	R.S.D. (%)	Accuracy (%)
450	438 ± 28	6.4	2.7	457 ± 41	8.9	1.5
600	617 ± 32	5.2	2.8	586 ± 20	3.5	2.3
750	728 ± 23	3.2	2.9	745 ± 10	1.3	0.7

[a] Values are mean of 10 determinations ± S.D.
[b] Mean of five determinations ± S.D.

3.3.4. Method 4

Since electrospraying of the internal standard with the matrix solution caused a high degree of analyte suppression, the concentration of TX was reduced from 0.25 to 0.05 mg/ml in α-CHCA. Even at this level suppression effects could not be prevented (Fig. 3D). However at lower levels the internal standard signal was not detected in a high number of mass spectra acquired during the scan. The high variation of the internal standard signal in the recorded mass spectra is shown in Fig. 3D (values vary from 0 to 53, which corresponds to an R.S.D. of 111%). This method like method 3 was not considered as suitable for the quantitative analysis of PX, since R.S.D. of 87% for the lowest and 28% for the highest point of the calibration curve were obtained ($n = 3$). The equation of the line in this case was $y = 0.0004x - 0.1340 (R^2 = 0.9970)$.

Comparing the three usable methods of incorporating the internal standard into the TLC plate, i.e. methods 2–4, the question arises why method 3 and 4 are not suitable for the quantification of PX by TLC–MALDI. A possible explanation lies in the MALDI process itself. The incorporation of the analyte into the matrix crystals is not a requirement, but improves the overall MALDI performance [33]. In method 3 and 4 the location of the internal standard and analyte molecules differ from each other. This highlights how important it is that analyte and internal standard are applied on the TLC plate in the same manner, by spotting (method 1) or by TLC development (method 2).

3.4. Method validation

The specificity of method 1 and 2 was tested on six blank TLC–MALDI samples respectively, and the corresponding mass spectra showed no interfering peaks between 330 and 340 Da (data not shown). Both methods exhibited linearity between the response (y) and the corresponding amount of PX (x), over the range of 400–800 ng. The mean correlation coefficient was 0.9992 ($n = 3$, method (1) or 0.9969 ($n = 4$, method (2), respectively. The LOD for PX was calculated as 16 ng and the LOQ as 53 ng (method 1). Method 2 showed two fold higher values: LOD = 39 ng and LOQ = 131 ng. The latter values are similar to the ones described for the quantitative HPTLC determination of PX (LOD = 40 ng, and LOQ = 150 ng) [5]. Table 1 shows the assay results for the analysis of three different quantities of PX. Good accuracy and precision was obtained for both methods.

4. Conclusions

Four methods for quantification by TLC–MALDI TOF MS have been compared. The drug Piroxicam could be successfully quantified by TLC–MALDI TOF MS when the internal standard was mixed into the mobile phase of the TLC analysis. The results of the method validation demonstrated that good; accuracy, precision, linearity and sensitivity could all be obtained using this method. The best precision for the standard calibration curve (400–800 ng Piroxicam) was obtained by incorporating the internal standard in the mobile phase (7–28% R.S.D., $n = 4$). The other methods investigated showed higher R.S.D. values up to 87% for the lowest point of the calibration curve and hence were not considered as suitable.

The simplicity of pre-developing the TLC plate with an appropriate internal standard to perform quantification in TLC–MALDI TOF MS makes this technique

particularly attractive to the pharmaceutical industry, where TLC is a standard analytical method for quality control samples (QC samples).

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