Thin-Layer Chromatography—Postsource-Decay Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry of Small Drug Molecules

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Abstract

The structural analysis of small drug molecules by directly coupling thin-layer chromatography (TLC) with postsource-decay (PSD) matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is reported. The applicability of this technique is shown using two examples: the TLC–PSD MALDI analysis of two representatives of nonsteroidal antiinflammatory drugs (tenoxicam and piroxicam) and the analysis of the pharmaceutically active compound UK-137,457 and one of its related substances UK-124,912. The matrices α-cyano-4-hydroxycinnamic acid (α-CHCA) and graphite are used to investigate the effect of the precursor ion selection on the TLC–PSD MALDI spectra of the drug molecules studied. Although α-CHCA enhances the $[M+H]^+$ ion formation graphite produces in general only sodium adducts. Structural differentiation of tenoxicam and piroxicam is possible only by selecting the sodium adduct of both drug molecules as precursor ions. In the case of the TLC–PSD MALDI analysis of UK-137,457 and its related substance UK-124,912 at the 1% level, the PSD spectra obtained in α-CHCA by selecting the protonated adduct of the small molecules as precursor ions shows distinguishable dissociation patterns containing structurally significant information.

Introduction

The introduction of matrix-assisted laser desorption/ionization (MALDI)–mass spectrometry (MS) as a soft ionization technique in the late 1980s by Karas and Hillenkamp (1) and by Tanaka et al. (2) has greatly helped to bring MS into biomolecular science. MALDI–MS enables the mass analysis of large, nonvolatile, and thermally labile compounds such as proteins, oligonucleotides, and synthetic polymers (3). In the 13 years since its introduction MALDI has become a standard method for the MS analysis of biological macromolecules.

In a typical MALDI analysis, the compound of interest is desorbed from a metal surface in an excess of matrix (typically an aromatic acid) using a pulsed nitrogen laser at 337 nm. The resulting gas-phase ions of the matrix and the analyte are usually determined by a time-of-flight (TOF) mass analyzer (4). Because a large portion of the molecular ions undergo intensive metastable decay in the TOF tube before they enter the mass analyzer, structural elucidation can be performed in a reflectron TOF-MS. Intact molecular ions, which gain sufficient internal energy during the desorption process, can release their excess energy by dissociation during their flight (i.e., by metastable decomposition). This process is called postsource decay (PSD) (5–9) in MALDI–TOF experiments. 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 the reflectron ("ion-mirror" part of the instrument) than lighter ions and thus mass-to-charge separation of the nondissociated precursor ion and the product ions is achieved.

The use of PSD in combination with MALDI MS has become a widely used technique for peptide sequencing since its introduction in the early 1990s (5). Compared with the large amount of work that has been carried out using PSD-MALDI for the analysis of biomolecules, little work has been conducted on the structural elucidation of low molecular weight compounds. Recently, Chen and Hercules (10) demonstrated the use of collision-induced dissociation (CID) PSD-MALDI MS for the analysis of pesticides. LeRiche et al. (11) compared the product ion spectra of 4-quinolone antibiotics produced by PSD- and PSD-CID-MALDI MS with electrospray ion-trap MS^n.

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The possibility of recording PSD-MALDI spectra directly from membranes was investigated by Guittard et al. (12). In this work glycolipid samples were separated on a thin-layer chromatographic (TLC) plate followed by heat transfer to a membrane.

An important area of ongoing research in MALDI is the examination of suitable matrices (13) to eliminate matrix interference in the analysis of small molecules such as pharmaceuticals. The use of hyphenated techniques, such as on-line coupling of column separations (e.g., liquid chromatography and capillary electrophoresis) and off-line coupling of planar separations (e.g., polyacrylamide gel electrophoresis and TLC) to MALDI, is also an area of current interest and has been the subject of two recent reviews (14,15).

In this study, the characterization of small drug molecules by their direct PSD-MALDI analysis on silica gel 60 F_{254} TLC plates is presented following TLC separation. Two *N*-heterocyclic carboxamide derivatives of benzothiazine-1,1-dioxide (tenoxicam and piroxicam) and the pharmaceutically active compound UK-137,457 and its related substance UK-124,912 were selected as model compounds. The effect of the precursor ion selection on the TLC–PSD-MALDI spectra was studied by using different matrices [α-cyano-4-hydroxycinnamic acid (α-CHCA) and graphite]. Possible structures of the product ions obtained from these investigations are proposed.

Experimental

Chemicals and materials

Tenoxicam ($C_{13}H_{11}N_3O_4S_2$) and piroxicam ($C_{15}H_{13}N_3O_4S$) were obtained from Sigma-Aldrich (Dorset, U.K.). UK-137,457 ($C_{31}H_{31}NO_5$), a pharmaceutical active compound, and one of its related substances UK-124,912 ($C_{27}H_{25}NO_3$) were supplied by Pfizer Global R&D (Sandwich, U.K.). The chemical structures of these compounds are shown in Figure 1. The matrices α-CHCA and graphite (1–2 µm) were purchased from Sigma-Aldrich. Methanol, acetonitrile, chloroform, and dichloromethane were all HPLC grade.

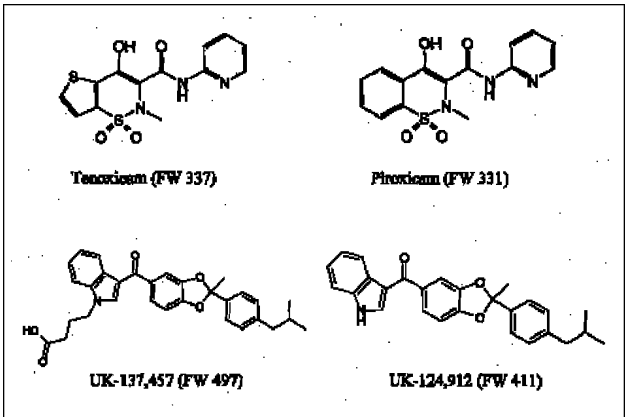


Figure 1. Structures of investigated compounds. The formula weight (FW) quoted is calculated using the monoisotopic atomic masses.

Sample preparation

Stock solutions of tenoxicam and piroxicam (2 mg/mL) were dissolved in dichloromethane. A mixture of both oxicam derivatives with a concentration of 1 mg/mL of each component was obtained by combining aliquots of both stock solutions.

Stock solutions of UK-137,457 (20 mg/mL) and UK-124,912 (1 mg/mL) were dissolved in acetonitrile. A mixture containing 10 mg/mL of UK-137,457 and 0.1 mg/mL of UK-124,912 was prepared by mixing both stock solutions in an appropriate ratio and diluting with acetonitrile to get the final concentrations.

TLC separation

Appropriate aliquots of the mixture were applied to an aluminum-backed TLC plate (10 × 10 cm) coated with 0.2-mm layers of silica gel 60 F_{254} (Merck, Darmstadt, Germany) using a 10-µL syringe. The development chamber was lined with filter paper and saturated with the corresponding mobile phase. For the separation of tenoxicam and piroxicam the mobile phase chloroform–methanol (9:1, v/v) (16) was used and saturated for 1 h before development of the plate. UK-137,457 was separated from its related substance UK-124,912 employing the mobile phase chloroform–methanol–glacial acetic acid (60:5:1, v/v/v), which was saturated overnight. The TLC plates were developed to a distance of 7.0 cm. After solvent evaporation at room temperature, the sample spots were visualized by illumination with UV light at λ = 254 nm.

Matrix application

Before the matrix was deposited on the silica-gel surface, a strip of the developed TLC plate (60 × 2 mm) was attached to a modified MALDI target with double-sided tape.

The organic matrix α-CHCA was applied to the silica-gel surface of the TLC plate by using an in-house modified commercial robotic *x*-*y*-*z*-axis motion system (PROBOT, BAI, Lautertal, Germany). Modifications to the instrument were conducted in order to use the apparatus as an electrospray deposition device. The alterations were as follows. A poly(tetrafluoroethylene) (PTFE) tube was used to transfer matrix solution from a 100-µL syringe to a Supelco (Poole, U.K.) coupling. The electrospray needle consisted of a 94-mm length of stainless steel capillary (0.5-mm o.d., 0.11-mm i.d.) (ASH Instruments, Macdesfield, U.K.), and the seal to the PTFE tube was made within the Supelco coupling. The electrospray needle was threaded through a stainless steel tee coupling ($^1/_{16}$-inch o.d.) (Snagelok, Warrington, U.K.) and a piece of stainless steel tubing (38-mm length, $^1/_{16}$-inch o.d., 0.75-mm i.d.) (Jones Chromatography, Hengoed, U.K.). A gas line was attached to the tee to provide nitrogen (2 bar) coaxial to the spray to assist in gas nebulization, if required.

Nebulizing gas was not used in these investigations because rapid solvent evaporation occurred. This led to a reduction in the extraction efficiency of the small drug molecules from the silica gel layer.

The syringe pump (Harvard microliter syringe pump, Harvard Scientific Apparatus, Edenbridge, U.K.) set at a flow rate of 0.6 mL/h delivered the matrix solution to the tip of the stainless steel capillary. In order to obtain a fine spray of the

matrix solution, approximately 2 kV was applied to the tee coupling using a high-voltage supply. The metal plate (7 × 15 cm) was earthed and placed 2 mm from the end of the electrospray needle. The plate was then moved across in front of the spray at a rate of 25 mm/s. Typically, an area of 60 × 2 mm on the TLC strip was covered with matrix crystals.

The inorganic matrix suspension of graphite was prepared by dispersing 80 mg graphite in 1 mL of methanol–ethylene glycol (1000:1, v/v). The suspension was homogenized by sonication for 15 min and then applied to the developed TLC strip using a 10-µL syringe. Typically, 30 µL of the graphite suspension was used to cover an area of 60 × 2 mm [electrospraying of matrix suspensions is not possible because of their particle nature (17)].

Instrumentation

An LT3 MS (SAI, Manchester, U.K.) equipped with a nitrogen laser (λ = 337 nm) was employed for this study. Modifications to the instrument and its software have been described previously by this group (18,19).

TLC–MALDI analysis was performed in linear or reflectron positive-ion mode with an accelerating voltage of 10 or 20 kV. Sixteen shots/spectrum were accumulated. The TLC strip was scanned over a distance of 60 mm, and spectra were recorded every 0.5 mm. A data set of 120 mass spectra were obtained, from which mass chromatograms of the studied compounds were constructed. The mass chromatograms enabled the location of the highest analyte signals on the TLC strip ("sweet spots"). It should be noted that the mass chromatograms presented in this study were smoothed.

TLC–PSD-MALDI analysis was performed at these sweet spots using an accelerating voltage of 20 kV and a focus mass applicable to the mass of the analyte. The gate was set to include the analyte of interest at ± 2 amu. Because this instrument used a harmonic mirror, full PSD spectra could be recorded without the need to combine small spectra segments.

Results and Discussion

TLC–MALDI analysis

Electrospray as a matrix deposition method for TLC–MALDI analysis was developed by this group (18,19). Scanning of a TLC strip and thus construction of mass chromatograms were possible with this application method because of a stable signal obtained and a homogeneous crystal layer of the matrix produced.

Figure 2A shows typical overlaid mass chromatograms of tenoxicam [$(M+H^+)$, *m/z* 338] and piroxicam [$(M+H)^+$, *m/z* 332] obtained from a TLC–MALDI experiment in reflectron positive-ion mode with an acceleration voltage of 20 kV. Two micrograms of both drug compounds were separated on a silica gel TLC plate, and the organic matrix α-CHCA [20 mg/mL in methanol containing 0.1% trifluoroacetyl (TFA)] was electrosprayed on the eluted TLC plate.

The phenomenon of "doughnut shaped" analyte spots normally occurs when polar solvents are used to apply the analyte spot on the TLC plate prior to TLC development (20). In TLC–MALDI analysis bimodal responses (as illustrated in Figure 2A) were a result of the electrospray deposition of the matrix, which was carried out in the polar solvent methanol. Both drug molecules have a solubility of < 1 mg/mL in methanol (21). This was necessary to ensure sufficient analyte extraction. However, solubility sufficient to give good analyte extraction from the silica-gel layer also caused some analyte spreading (indicated as peak broadening in Figure 2A).

Although the limit of detection of piroxicam is estimated to be 39 ng when α-CHCA is employed as the MALDI matrix, 2-µg quantities of both drug compounds were chosen in order to allow for scanning of the TLC plate and PSD analysis to be carried out on the same sample. A high analyte concentration was particularly required when graphite was used as the matrix, because fast signal decrease occurred when the laser was targeted on the same TLC position.

The generation of an impurity profile of UK-137,357 is presented in Figure 2B. The overlaid mass chromatograms of the parent compound UK-137,457 [$(M+H)^+$, *m/z* 498] and its related substance UK-124,912 [$(M+H)^+$, *m/z* 412] at the 1% level were constructed from a TLC–MALDI analysis in linear

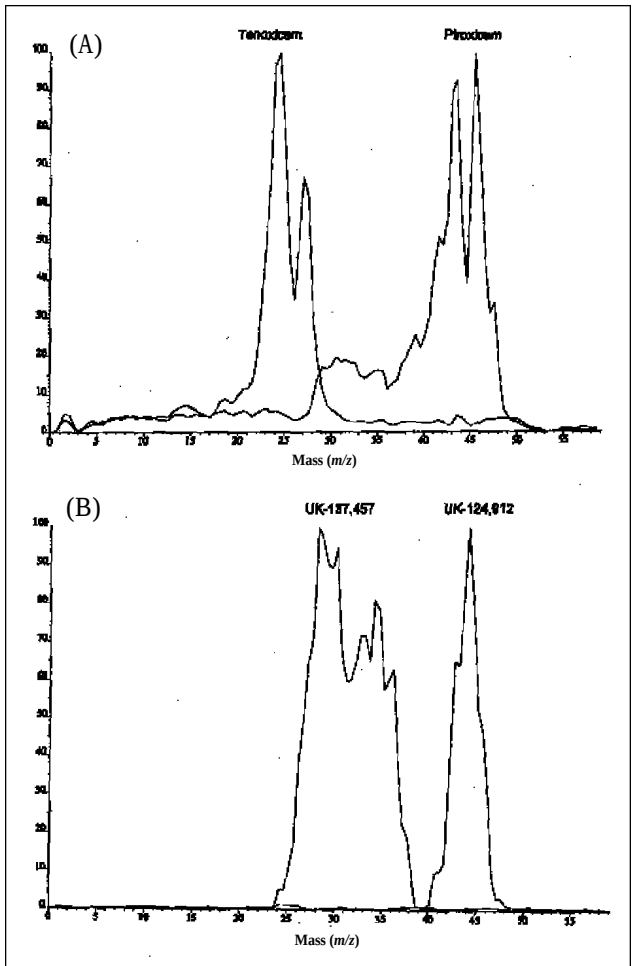


Figure 2. Overlaid mass chromatograms from the TLC–MALDI determination of (A) tenoxicam and piroxicam (2 µg of each component) and (B) UK-137,457 (100 µg) and its known related substance UK-124,912 at the 1% level. In both analyses the small molecules were separated on a silica gel TLC plate and α-CHCA was used as the matrix (as described in the "Experimental" section).

positive ion mode using an acceleration voltage of 10 kV. One hundred micrograms of the parent compound was separated from 1 µg of the related substance on a silica gel TLC plate, and the organic matrix α-CHCA (20 mg/mL in acetonitrile–water containing 0.1% TFA in the volume ratio 7:3) was electrosprayed on the eluted TLC plate. In order to obtain a sufficient signal of the related substance for the following PSD analysis, a 1% level was chosen even though the sensitivity of the related substance was below 1 ng. With a quantity of 100 µg of the parent compound, the TLC plate was readily overloaded (as indicated in Figure 2B).

TLC–PSD-MALDI analysis

The mass chromatograms obtained from the TLC–MALDI analysis (Figure 2) enabled the location of the highest analyte signals on the TLC strip, and TLC–PSD-MALDI analyses were performed at these positions.

Two matrices were chosen for the TLC–PSD-MALDI analysis of tenoxicam and piroxicam, the organic matrix α-CHCA, and the particle suspension matrix graphite. α-CHCA was classified as a "hot" matrix in MALDI because of the high dissociation level induced (22). The use of graphite for PSD experiments has not been previously reported. However, previous studies of graphite for the TLC–MALDI analysis of tetracylcines showed high in-source fragmentation rates in the linear positive- and negative-ion mode (17).

The recorded TLC–PSD-MALDI spectrum of tenoxicam in α-CHCA is presented in Figure 3A. The protonated molecular species of tenoxicam at *m/z* 338 was selected as the precursor ion. An interference peak of α-CHCA (indicated as M* in the spectrum) was observed at *m/z* 335. α-CHCA with a molecular weight of 189 amu formed a dimer $[2M\text{-}CO_2+H]^+$ at *m/z* 335, which passed through the ion gate and entered the mass analyzer.

For piroxicam the protonated molecular species at *m/z* 332 was chosen as the precursor ion. The corresponding TLC–PSD-MALDI spectrum of piroxicam in α-CHCA is shown in Figure 3B. The interfering peak of the matrix (indicated as M*) was observed in low abundance.

In order to ensure that the detected product ions in Figures 3A and 3B were not arising from dissociated ions of α-CHCA, TLC–PSD-MALDI analysis was carried out on TLC plate positions at which no analyte was located (i.e., the matrix only was present in this position). No product ions with *m/z* values (shown in Figures 3A and 3B) were detected.

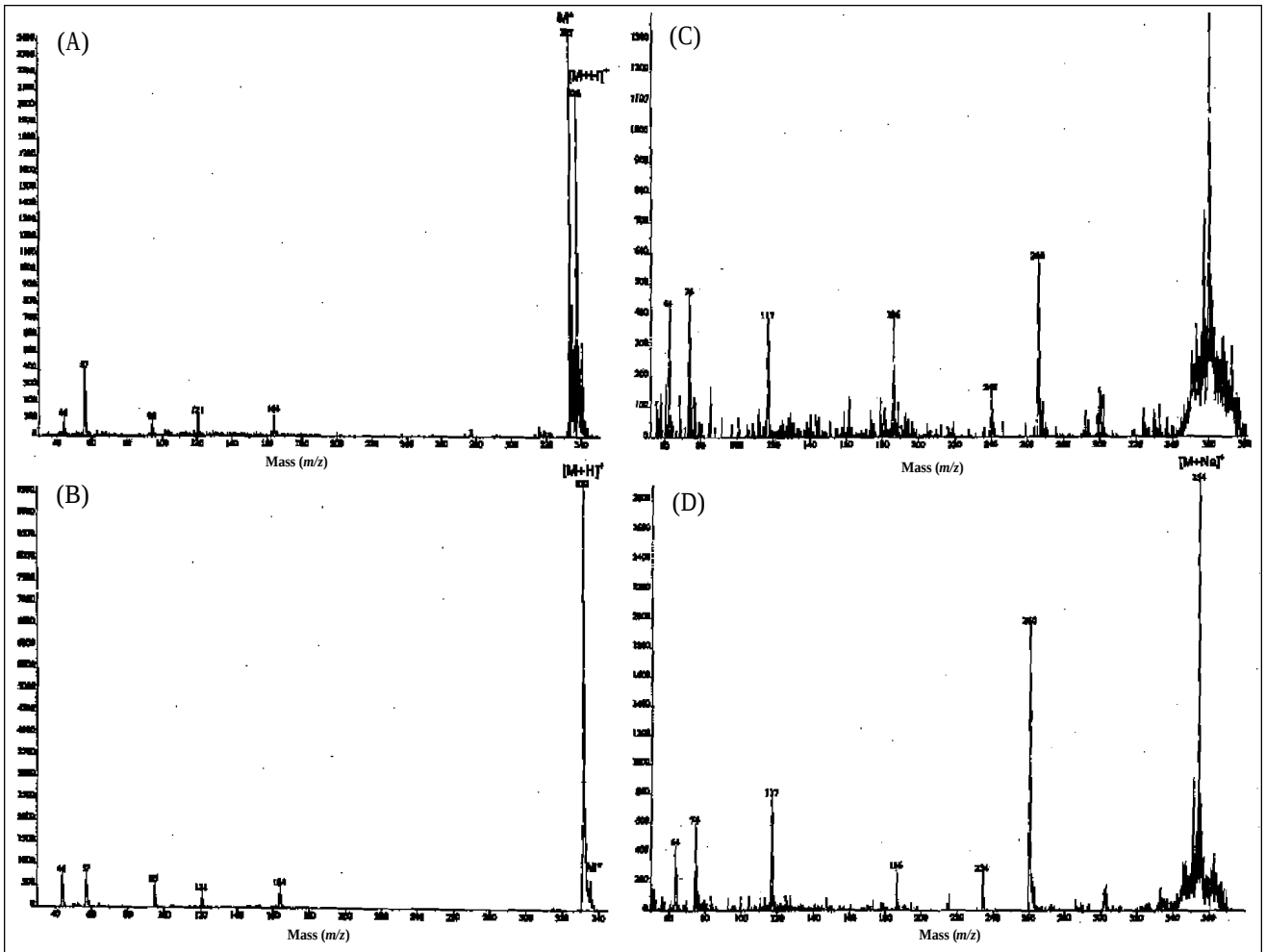
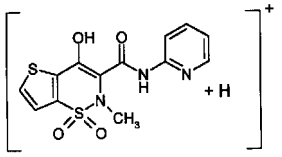
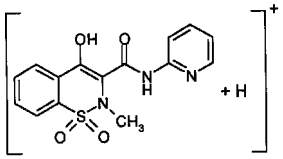
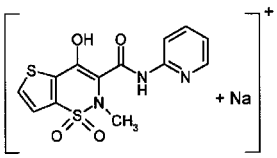
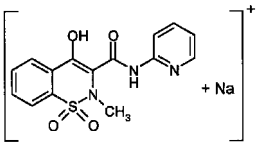
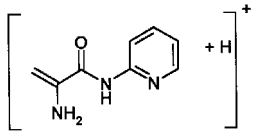
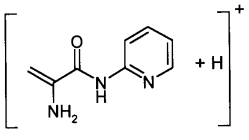
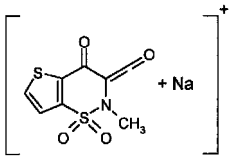
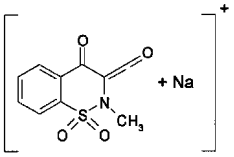
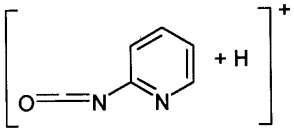
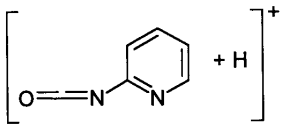
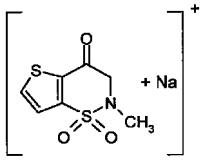
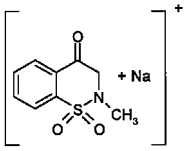
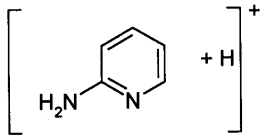
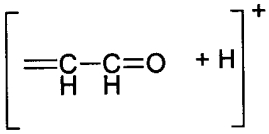
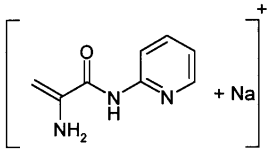
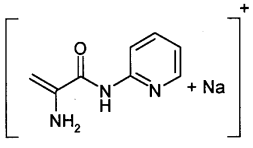
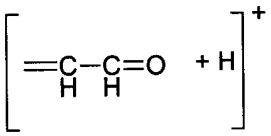
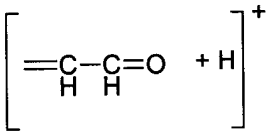
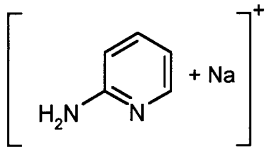
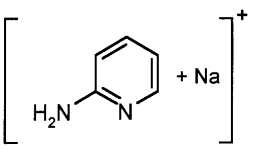
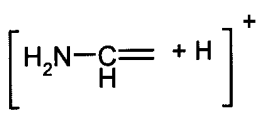
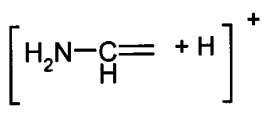
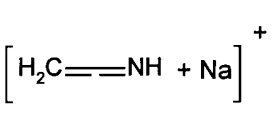
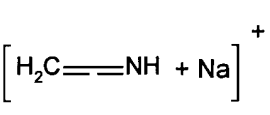


Figure 3. TLC–PSD-MALDI spectra of tenoxicam (2 µg) and piroxicam (2 µg) separated on silica gel TLC plates using different matrices. Mass spectra of (A) tenoxicam and (B) piroxicam were acquired using α-CHCA as the matrix, and mass spectra of (C) tenoxicam and (D) piroxicam were acquired using graphite as matrix.

The oxicam derivatives tenoxicam and piroxicam form mainly sodium adduct ions in TLC–MALDI analysis using graphite as the matrix because sodium is believed to be a major contaminant in graphite. Thus TLC–PSD-MALDI analysis in graphite was performed on the $[M+Na]^+$ ion of tenoxicam at *m/z* 360 and piroxicam on the $[M+Na]^+$ ion at *m/z* 354. The resulting TLC–PSD-MALDI spectra of both drug molecules are shown in Figures 3C and 3D, respectively. A comparison of the TLC–PSD-MALDI spectra of tenoxicam and piroxicam obtained in graphite (Figures 3C and 3D) with α-CHCA (Figures 3A and 3B) shows that a lower noise level was obtained when the latter matrix was employed.

In order to improve the mass accuracy obtained on a TLC plate, which is limited by the porosity of the silica-gel surface, the instrument was recalibrated using the selected precursor ion in the positive reflectron ion mode at the TLC position from which the TLC–PSD-MALDI spectra were recorded. Comparison of the *m/z* values of the product ions obtained on the

Table I. Proposed Structures of the Product Ions Observed in the TLC–PSD-MALDI Mass Spectra of Figure 3*

Column 1		Column 2	
Tenoxicam	Piroxicam	Tenoxicam	Piroxicam
			
(*m/z* 338)	(*m/z* 332)	(*m/z* 360)	(*m/z* 354)
			
(*m/z* 164)	(*m/z* 164)	(*m/z* 266)	(*m/z* 260)
			
(*m/z* 121)	(*m/z* 121)	(*m/z* 240)	(*m/z* 234)
			
(*m/z* 95)	(*m/z* 95)	(*m/z* 186)	(*m/z* 186)
			
(*m/z* 57)	(*m/z* 57)	(*m/z* 117)	(*m/z* 117)
			
(*m/z* 44)	(*m/z* 44)	(*m/z* 64)	(*m/z* 64)

* Column 1 shows the proposed product ions when the $[M+H]^+$ ion of both drug molecules was chosen as the precursor ion, and column 2 when the $[M+Na]^+$ ion was used.

silica gel surface with the normal stainless steel surface showed no differences.

Possible structures for the product ions of tenoxicam and piroxicam are proposed in Table I. It can be clearly seen that the selection of the protonated species as a precursor ion for tenoxicam and piroxicam in the organic matrix α-CHCA produced the same product ions for both drug molecules because dissociation presumably occurs from the thiophene or benzene group, which represented the only structural difference between both molecules (Table I, column 1).

In contrast, the selection of the sodium adduct as a precursor ion for both of the oxicam derivatives in the experiments conducted using the particle suspension matrix graphite generated distinguishable dissociation patterns. Graphite had advantages for the TLC–PSD-MALDI analysis of these compounds; no matrix interference peaks were detected (Figures 3C and 3D) and also the $[M+Na]^+$ ions of tenoxicam and piroxicam were detected with high signal intensities.

The addition of sodium chloride to α-CHCA was tried in order to produce sodium adducts of the drug molecules. However, the generated sodium adducts of tenoxicam and piroxicam were of low abundance and the protonated ions were still the dominant species. Despite this, when the sodium adducts were selected as precursor ions for PSD analysis, the same dissociation patterns were obtained as if graphite would have been used as the matrix. This proves that the degree of dissociation of the drug molecules studied does not depend on the choice of matrix, but rather on the selection of the precursor ion.

All of the product ions observed (Table I column 2) were believed to be sodium adducts. Dissociation was believed to occur from both sides of the drug molecules, the thiophene or

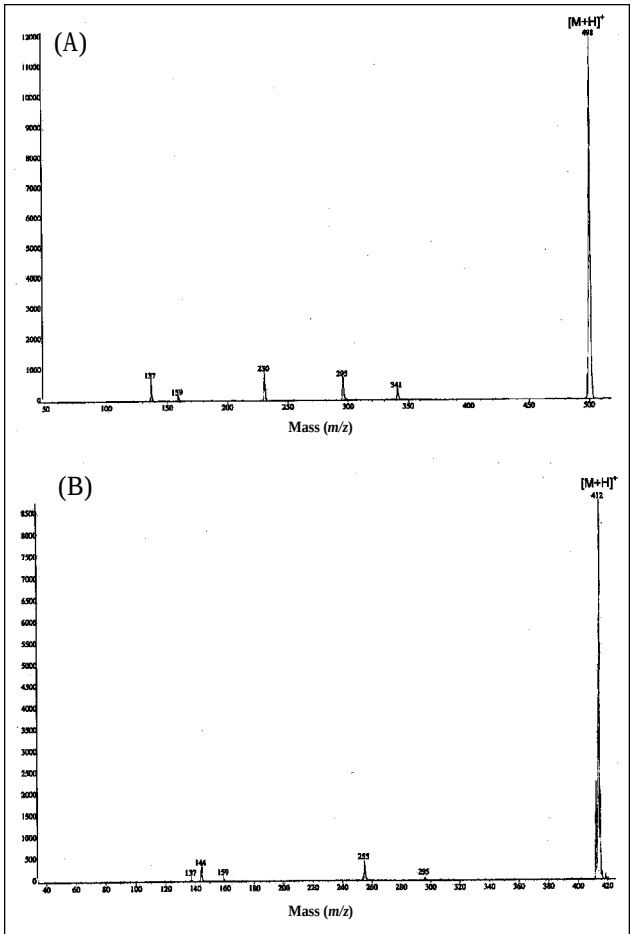
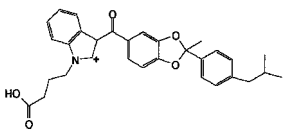
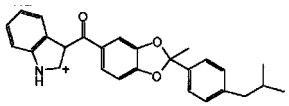
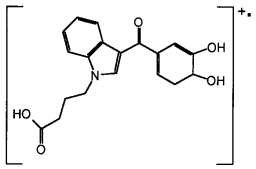
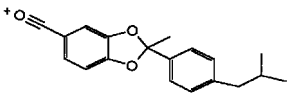
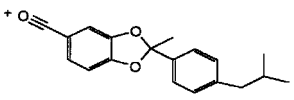
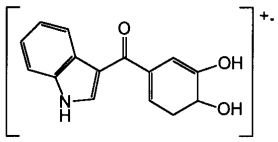
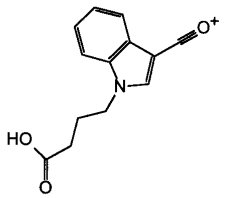
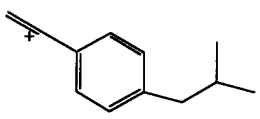
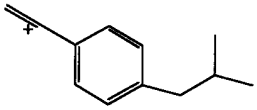
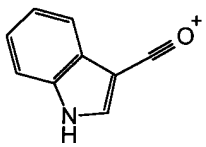
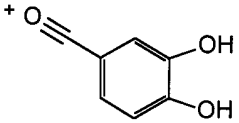
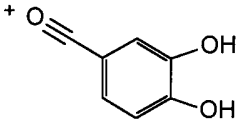


Figure 4. TLC–PSD-MALDI spectra of (A) UK-137,457 (100 µg) and (B) its related substance UK-124,912 at the 1% level. The related substance UK-124,912 (1 µg) was separated on a silica gel TLC plate from the parent compound and α-CHCA was used as the matrix.

Table II. Proposed Structures of the Product Ions Observed in the TLC–PSD-MALDI Mass Spectra of Figure 4

UK-137, 457	UK-124, 912
 (*m/z* 498)	 (*m/z* 412)
 (*m/z* 341)	 (*m/z* 295)
 (*m/z* 295)	 (*m/z* 255)
 (*m/z* 230)	 (*m/z* 159)
 (*m/z* 159)	 (*m/z* 144)
 (*m/z* 137)	 (*m/z* 137)

the benzene-group, and the pyridine-group.

For the TLC–PSD-MALDI analysis of UK-137,457 in α-CHCA, the protonated molecular species at *m/z* 498 was selected as the precursor ion. The corresponding PSD spectrum is shown in Figure 4A. The $[M+H]^+$ ion of the related substance UK-124,912 was detected at *m/z* 412 and was used as a precursor ion for PSD analysis (Figure 4B). In both TLC–PSD-MALDI spectra, α-CHCA did not show any interfering peaks. Possible structures of the recorded product ions are summarized in Table II. The important species were those of *m/z* 230 and 341 for UK-137,457 and *m/z* 144 and 255 for UK-124,912. As can be seen from Table II, the difference in the *m/z* value of 86 between corresponding ions was the relative molecular mass of the side chain group of the pyrrole ring (Figure 1) of UK-137,457. This allowed for the structural identification of UK-124,912.

Conclusion

TLC–PSD-MALDI spectra of the drug analogs tenoxicam and piroxicam and the pharmaceutically active compound UK-137,457 and its related substance UK-124,912 have been successfully obtained, and the effect of the precursor ion selection on this technique was evaluated. The extent of PSD of these small molecules was determined by the selection of the precursor ion. A high degree of PSD activation and distinguishable product ions were only obtained for tenoxicam and piroxicam when the sodium adduct was chosen as the precursor ion. α-CHCA showed the disadvantage of generating matrix ions, which could not be eliminated from the PSD spectra because of the low resolution of the ion gate. In the TLC–PSD-MALDI analysis of UK-137,457 and its related substance UK-124,912 in α-CHCA, differentiation in the PSD spectra was achieved when the protonated ion of both molecules was selected. It was possible to obtain TLC–PSD-MALDI data from UK-12912 at the 1% level, which allowed for structural characterization.

Acknowledgments

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