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Two-Dimensional Ultra-Thin-Layer Chromatography and Atmospheric Pressure Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry in Bioanalysis

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The feasibility of ultra-thin-layer chromatography (UTLC) and atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry (AP-MALDI-MS) for bioanalysis was studied with benzodiazepines as model substances in human urine. Two-dimensional (2D) UTLC was shown to be an efficient technique for the separation of benzodiazepines. Separations occurred in 4−12 min, and the separated compounds were identified by AP-MALDI-MS. The limits of detection with AP-MALDI-MS and AP-MALDI-MS/MS were in the picomole range and thus low enough for bioanalysis. The applicability of the 2D UTLC-AP-MALDI-MS was demonstrated in detection of metabolites with an authentic biological urine sample.

The combination of modern high-performance thin-layer chromatography (HPTLC) with automated sample application and densitometric scanning makes this sensitive and reliable technique highly suitable for qualitative and quantitative analysis in pharmaceutical, environmental, toxicological, and forensic applications.[1−4] TLC plates are disposable, and there is no memory effect as may occur in LC. TLC is also a fast, simple, and inexpensive method that allows the analysis of many samples simultaneously and the possibility to use a number of nondestructive detection methods and appropriate derivatization reagents in sequence. The disadvantages of TLC compared with LC or GC are, however, lower resolution and higher limits of detection (LODs), although the separation efficiency of TLC can be significantly improved by applying two-dimensional (2D) elution.[4−7] 2D TLC is simple to carry out and the technique has already been successfully applied, for example, to the analysis of drugs and plant extracts.[5−7]

A new planar chromatographic method, ultra-thin-layer chromatography (UTLC), was recently introduced by Hauck et al.[8] Relative to conventional or high-performance (HP) TLC methods, UTLC provides faster separation times, lower solvent consumption, and lower detection limits with UV and MS.[9,10] One weakness of UTLC compared with HPTLC is the reduced resolution caused by the shorter elution distances and smaller overall specific adsorption surface area.[9,10]

The specificity of UV or fluorescent detection alone may be insufficient for complex bioanalysis, and different ways to combine TLC and MS have been explored during the past few years.[10−18] Van Berkel and co-workers have introduced interesting new couplings of TLC with different ionization techniques, for example, atmospheric pressure chemical ionization (APCI),[12,13] electrospray ionization (ESI),[14,15] and desorption electrospray ionization (DESI).[16,17] We, in turn, have introduced a combination of UTLC and atmospheric pressure matrix-assisted laser desorption ionization (AP-MALDI)-MS,[10] and most recently a coupling of UTLC and DESI techniques.[18] Nevertheless, MALDI-MS continues to be a frequently used method for direct TLC-MS analysis.[19−23] Choosing the right MALDI matrix is essential for successful TLC-MALDI-

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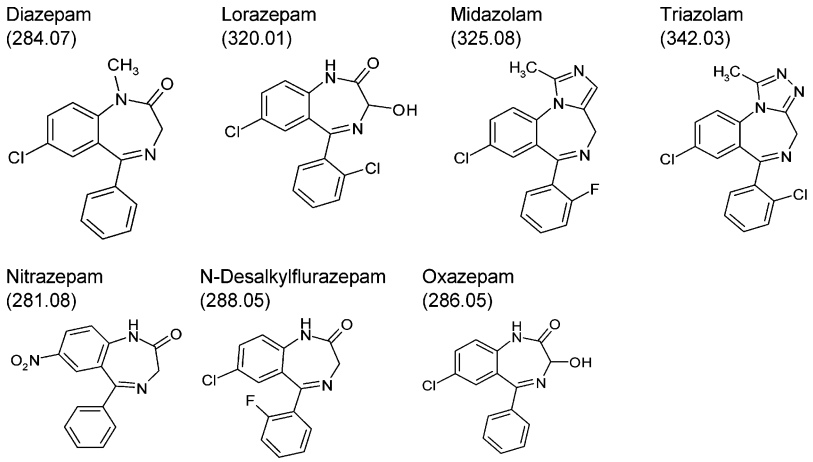

Figure 1. Structures and molecular weights of the benzodiazepines examined.

MS analysis. Many matrixes cause interfering mass peaks at low m/z range, but these interferences can be minimized through use of a suitable analyte-to-matrix molar ratio[24,25] or the matrix that produces a low background.[26−28] Moreover, though desorption from the irregular surface of TLC plates with a vacuum MALDI time-of-flight (TOF) instrument has detrimental effect to repeatability of the measured m/z values,[10,29,30] the problem can be overcome by using an AP-MALDI-ion trap-MS[10] or a MALDI-Fourier transform (FT)-MS.[31]

In this study, we present for the first time a two-dimensional UTLC-AP-MALDI-MS for analysis of biological samples. The feasibility of the method was demonstrated in qualitative analysis of benzodiazepines in authentic human urine sample collected after ingestion of diazepam. The influence of the urine matrix on the separation and repeatability was evaluated and is discussed.

EXPERIMENTAL SECTION

Reagents. Reference standards (Figure 1) for benzodiazepine were midazolam (Roche, Basel, Switzerland), triazolam, diazepam, oxazepam, *N*-desalkylflurazepam, nitrazepam, and lorazepam (Sigma-Aldrich, St. Louis, MO).

Blank urine was obtained from a healthy volunteer and stored at −20 °C until use. *Helix pomatia* (containing 112400 IU of β-glucuronidase per mL) used for enzymatic hydrolysis of the urine sample was obtained from Sigma-Aldrich.

α-Cyano-4-hydroxycinnamic acid (α-CHCA), used as the matrix compound in MALDI-MS analysis, was purchased from Fluka Chemie (Buchs, Switzerland). Bismuth subnitrate (Merck, Darmstadt, Germany) and potassium iodide (Riedel-de Haën, Seelze, Germany) were used in the preparation of the Dragendorff reagent. Compounds for the buffer solution were ammonium carbonate (University Pharmacy, Helsinki, Finland), ammonium acetate (Riedel-de Haën), and sodium acetate (Merck).

All organic solvents were of analytical or chromatographic grade. Methanol and toluene were purchased from J.T. Baker (Deventer, Holland). Acetone was from Merck, dichloromethane from Mallinckrodt (Deventer, Holland), acetonitrile from Rathburn (Walkerburn, Scotland), and ethanol from Altia (Rajamäki, Finland). Trifluoroacetic acid, glacial acetic acid, and 25% ammonia solution were from Acros Organics (Geel, Belgium), Bang&Bonsomer (Helsinki, Finland), and J.T. Baker, respectively. Water was Milli-Q purified (Millipore, Molsheim, France).

Samples and Solutions. All stock solutions of the benzodiazepine reference compounds were prepared by dissolving the compound to a concentration of 1 mg/mL in acetone/ethanol (50:50 v/v). The same solvent was used to dilute the stock solutions to appropriate concentrations. Spiked urine samples were prepared by adding 10 μL of each benzodiazepine solution (10 μg/μL) to the mixture of 1 mL of blank urine and 2 mL of ammonium carbonate buffer solution (0.01 M; pH 9.3). An authentic urine sample was collected from a healthy volunteer 53 h after intake of 10 mg of diazepam.

The stock solution of the MALDI matrix (13.3 mg/mL) was prepared by adding 20 mg of α-CHCA to 1.5 mL of methanol/acetonitrile solution (1/2 v/v) containing 0.1% trifluoroacetic acid. The working solution of the matrix was prepared by diluting the stock solution with acetonitrile to a concentration of 9.4 μg/μL (10 nmol/μL).

For Dragendorff reagent,[32] solution A was prepared by dissolving 0.85 g of basic bismuth subnitrate in a mixture of 10 mL of glacial acetic acid and 40 mL of water at 50 °C and solution B by dissolving 8 g of potassium iodide in 30 mL of water. The stock solution was obtained by mixing solutions A and B (1:1), and finally, for the spray solution, 1 mL of the stock solution was mixed with 2 mL of glacial acetic acid and 10 mL of water.

Solid-Phase Extraction. Solid-phase extraction (SPE) was carried out by using an Oasis HLB 1 cm^3 (30 mg) SPE column (Waters Oasis, Milford, MA), which was conditioned and washed

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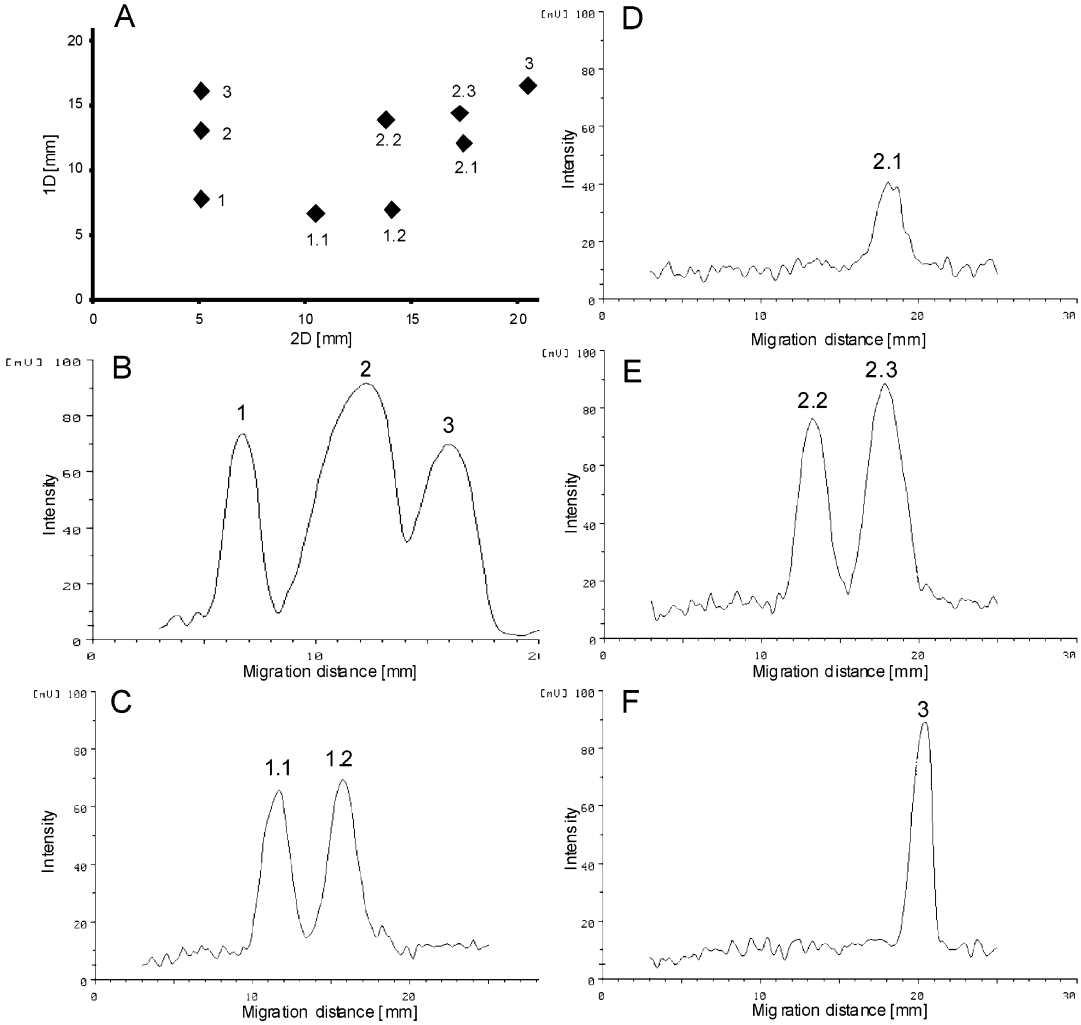

Figure 2. (A) Illustrative presentation of benzodiazepine zones on a UTLC plate after 1D and 2D separations. (B)−(F) UV densitograms of separated benzodiazepines measured from a UTLC plate after 1D separation (B) and 2D separation (C)−(F).

twice with 1 mL of MeOH, 1 mL of water, and then 1 mL of ammonium carbonate buffer (0.01 M, pH 9.3). Blank urine or urine spiked with benzodiazepines (3 mL) was added. The column was washed twice with 1 mL of water and 250 μL of the mixture ACN/H_2O (10:90 v/v) and dried under vacuum for 5 min. The extraction was carried out with 1 mL of MeOH, and the extraction solution was evaporated to dryness with a vacuum apparatus (GeneVac Technologies HT4-Series II, Ipswich, England). The residue was reconstituted to 1 mL of acetone/ethanol (50:50 v/v).

The SPE recoveries were measured with urine samples spiked with benzodiazepines (200 μg/mL) before and after the extraction. The recovery samples were analyzed with a HP 1100 series liquid chromatograph (Agilent GmbH, Waldbronn, Germany) using a UV detector at wavelength 254 nm. Eluent A was 50 mM ammonium acetate in water (pH 4.0) and eluent B was methanol. The sample solution (20 μL) was injected with an autosampler, via a guard column (LiChroCART 4-4, Purospher RP-18 e with particle size of 5 μm, Merck), to a 125 × 3 mm LichroCART Purospher RP-18 e column with particle size of 5 μm (Merck). The flow rate of the mobile phase was 0.6 mL/min. The gradient program was 0−10 min 5−90% B, 10−15 min 90% B, and 15−18 min 90−5% B. The column was stabilized for 7 min between analyses. HPLC separations were performed at room temperature.

Enzymatic Hydrolysis of the Urine Matrix. Two molar sodium acetate buffer (0.2 mL) (pH 4.5) and *Helix pomatia* corresponding to 5500 IU were added to 1 mL of authentic urine. The mixture was incubated at 56 °C for 1.5 h, cooled, and centrifuged (10 min) at 5000*g*. Supernatant and 2 mL of 0.01 M ammonium carbonate buffer (pH 9.3) were vortex-mixed, after which the sample was purified by SPE as described above. The method was selected on the basis of earlier studies on diazepam and its metabolites.[33,34]

UTLC Method. Planar chromatography was done on monolithic UTLC plates (glass support) of 3.6 × 6 cm (Merck, Darmstadt, Germany). The plates were washed once with methanol before sample application. Sample solutions were sprayed as thin rectangular bands onto the adsorbent in amounts of 1−10 μL (depending on the sample concentration) with a Linomat IV (Camag, Muttenz, Switzerland) at a flow rate of 1 μL/10 s. Bands were 3 mm long and 4 mm apart. The total amount of analytes on the plate was between 0.1 pmol and 1 nmol.

The mobile phase composition was optimized separately for the 1D and 2D separations. Dichloromethane/acetone (93 + 7

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Table 1. LODs (S/N = 3) for the Benzodiazepines Measured from the Developed UTLC Plate with UV, MS, and MS/MS Detection

	UV (pmol)		MS (pmol)		MS/MS (pmol)	
compd	standard	spiked in urine	standard	spiked in urine	standard	spiked in urine
midazolam	42	75	4.6	8.6	0.2 (291)	0.4 (291)
diazepam	32	38	11	7.9	1.3 (257)	1.4 (222)
lorazepam	64	56	22	52	6.4 (303)	6.4 (303)
oxazepam	40	58	67	97	2.0 (269)	1.3 (269)
N-desalkyl-flurazepam	48	71	8.3	91	7.9 (226)	84 (261)
triazolam	226	95	2.0	9.7	6.0 (308)	1.7 (308)
nitrazepam	42	57	7.5	97	19 (236)	21 (236)

v/v) was used as the final mobile phase for 1D separation and toluene/acetone/ethanol/25% ammonia solution (70 + 20 + 3 + 1 v/v/v/v) for 2D separation. The plates were developed in a saturated chamber to the distance of 2 cm (in both dimensions). The developing time in one dimension was 2−6 min, and the total time was 4−12 min. After separation, the plates were scanned with a Camag TLC Scanner II (Muttenz, Switzerland) controlled by the CATS 3.17 program at $\lambda = 254$ nm (D_2 lamp). The densitometric measurements were performed in absorption and reflection modes. For visual detection of benzodiazepines on the plate the analytes were derivatized by spraying the plate with Dragendorff reagent.

MALDI Instrumentation. For MALDI measurements, 1 μL of the working solution of the α-CHCA matrix was sprayed over the separated sample zone with a Linomat IV applicator. The concentration of the α-CHCA solution was 9.4 μg/μL so that the total amount of α-CHCA on the plate was 10 nmol. After application of the matrix, the UTLC plate was cut to match the face of an in-house-modified AP-MALDI target plate and was attached to it with double-sided conductive tape. The AP-MALDI mass spectrometry system consisted of an AP-MALDI ion source (Agilent Technologies) combined with an LC-MSD-Trap-XCT-Plus ion trap instrument (Agilent Technologies). The AP-MALDI interface has been described in detail by Doroshenko et al.[35] A nitrogen laser at 337 nm (10 Hz) was focused on the sample zone of the plate, the size of the laser spot being 0.5 mm.[35] The laser pulse energy was adjusted with an attenuator to 8.5 (arbitrary unit), providing an estimated pulse energy of 264 μJ. The ions that formed were directed to the ion trap via an extended capillary (1500 V). Nitrogen was used as drying gas with a flow rate of 6 L/min and temperature of 150 °C. The ion trap parameters were as follows: the accumulation time was 200 ms, the averages were 10, and the rolling averaging was off. The voltages of the skimmer and capillary exit were 70 and 265 V, respectively. The mass spectra were recorded in the range of m/z 100−500. For MS/MS measurements, the cutoff value was set to m/z 50 and the fragmentation amplitude was 1.0−2.0. Other parameters were the same as in the MS mode.

RESULTS AND DISCUSSION

UTLC Separation and UV/Vis Detection. Six reference standards of benzodiazepines were used in the development of the 2D UTLC method. The 1D separation was performed with dichloromethane/acetone (eluent 1) and the 2D separation with toluene/acetone/ethanol/25% ammonia solution (eluent 2). The selection of the solvents was based on earlier studies in which benzodiazepines were separated by TLC.[36−38] The final composition of the eluents was optimized to achieve acceptable separation.

Table 2. Repeatabilities of Migration Distances (mm) of Benzodiazepine Standards and Benzodiazepines Spiked in Urine ($n = 5$) after 1D and 2D Separations as Mean, Standard Deviation (SD), and Relative Standard Deviation (RSD %)[a]

	diaze-pam	nitraze-pam	oxaze-pam	*N*-desalkyl-flurazepam	midazo-lam	triazo-lam
Reference Standards						
y-Axis (in mm)						
mean	16.6	14.4	13.9	12.1	6.9	6.7
SD	0.4	1.0	1.0	1.2	0.4	0.2
RSD %	2.3	6.7	7.3	9.9	5.4	3.7
x-Axis (in mm)						
mean	20.5	17.3	13.8	17.5	14.1	10.5
SD	1.3	0.9	0.7	0.8	1.4	0.7
RSD %	6.2	5.4	4.9	4.8	9.7	7.2
Spiked in Urine						
	diaze-pam	nitraze-pam	oxaze-pam	*N*-desalkyl-flurazepam	midazo-lam	triazo-lam
y-Axis (in mm)						
mean	16.1	13.6	13.0	11.6	6.3	6.2
SD	0.2	1.0	0.8	1.1	0.4	0.4
RSD %	1.2	7.1	6.0	9.2	6.3	6.5
x-Axis (in mm)						
mean	19.1	16.5	13.7	16.4	12.5	9.0
SD	0.9	1.0	1.0	0.9	1.3	1.0
RSD %	4.8	6.4	7.5	5.2	10.4	11.7

[a] The *y*- and *x*-axes represent the migration distances on the UTLC plate after 1D separation (*y*-axis) and after 2D separation (*x*-axis).

The commercial UTLC plates lack a fluorescent indicator, and to obtain adequate data to specify the exact location of the benzodiazepines on the 2D eluted plate, we tested dyeing with Dragendorff reagent[32] and UV densitometric detection. With the Dragendorff reagent the benzodiazepines could be rapidly visualized as derivatives down to 100 and 500 pmol (i.e., 30−150 ng), a level adequate for method development but too high for bioanalysis.

In testing the feasibility of UTLC-UV densitometry for the detection of benzodiazepines after 2D separation, we scanned the entire eluted area (20 × 20 mm) with equidistant spacing of 1 mm. The UV densitogram of the first dimension showed three zones (1, 2, and 3) (Figure 2A and B). After 2D separation all six standard compounds were separated (Figure 2A and C−F). Zone 1 was separated into two zones (1.1 and 1.2) and zone 2 into three zones (2.1, 2.2, and 2.3). Note that the zones 2.1 and 2.3 were actually separated already in 1D separation, but the zone 2.2 was situated between the zones 2.1 and 2.3 and, therefore, only one broad peak 2 was seen after 1D separation (Figure 2B). After 2D development, the zone 2.2 was separated from the zones 2.1 and 2.3 and the 1D separation of 2.1 and 2.3 became visible, although they had the same R_F values in 2D separation. Zone 3

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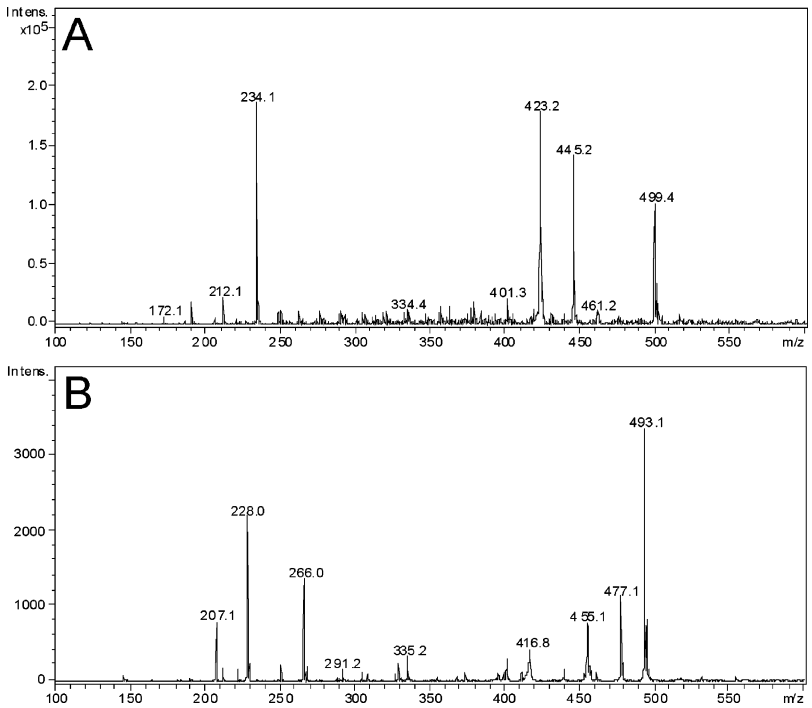

Figure 3. MS spectra of neat α-CHCA matrix without (A) and with the Dragendorff reagent (B). The amount of the matrix was 10 nmol.

Table 3. Main Molecular, Fragment, and Product Ions of Selected Precursor Ions of Analytes Measured by UTLC-AP-MALDI-MS and UTLC-AP-MALDI-MS/MS before Separation[a]

	MS *m/z* (rel abund)			precursor	MS/MS
compd	$[M + H]^+$	$[M + Na]^+$	other ions	$[M + H]^+$	*m/z* (rel abund) [product ion]
midazolam	326 (100)	-		326	291 (100) $[M + H - CI]^+$; 244 (29); 209 (2)
diazepam	285 (100)	307 (13)	257 (7)	285	257 (100) $[M + H - CO]^+$; 222 (60) $[M + H - CO - CI]^+$ 228 (40) $[M + H - CO - CH_2NH]^+$; 193 (20); $[M + H - CO - CH_2NH - CI]^+$182 (30); 154 (60)
lorazepam	321 (77)	343 (100)	303 (19); 275 (11)	321	303 (100) $[M + H - H_2O]^+$; 275 (50) $[M + H - H_2O - CO]^+$; 208 (10)
oxazepam	287 (100)	309 (90)	269 (23); 241 (15)	287	269 (100) $[M + H - H_2O]^+$; 241 (20) $[M + H - H_2O - CO]^+$
N-desalkyl-flurazepam	289 (100)	311 (11)	261 (6)	289	261 (100) $[M + H - CO]^+$; 226 (80) $[M + H - CO - CI]^+$; 140 (94)
triazolam	343 (100)	365 (30)		343	308 (100) $[M + H - CI]^+$; 315 (25); 279 (4)
nitrazepam	282 (100)		236 (21)	282	236 (100) $[M + H - NO_2]^+$

[a] Sample amount was 1 nmol and matrix amount 10 nmol.

included only one compound (3). On the basis of the analysis with the individual standards, zone 1.1 was assigned to triazolam, 1.2 to midazolam, 2.1 to *N*-desalkylflurazepam, 2.2 to oxazepam, 2.3 to nitrazepam, and 3 to diazepam. The UV densitometer provided lower limits of detection, about 30−100 pmol (Table 1), and higher specificity than the Dragendorff reagent. However, the localization of the separated zones after 2D separation requires relatively time-consuming (about 5−10 min) scanning of the entire eluted area of the UTLC plate.

The suitability of 2D UTLC-UV for the determination of benzodiazepines directly from urine without a prior purification was studied with a sample of human urine spiked with diazepam (500 pmol). After 2D separation the zone of diazepam (zone 3) was broader but located at the same position on the plate as the zone of diazepam of the pure reference standard. The zone was broader because the standard solution was prepared in acetone/ethanol (50:50 v/v) and acetone/ethanol produces a narrower application zone on the plate than aqueous urine sample. To achieve narrower zones and good resolution, the benzodiazepines in urine samples were extracted by SPE, evaporated, and reconstituted with acetone/ethanol. The recoveries after the purification procedure, measured by HPLC-UV, were between 90.3 and 98.5% and are in accordance with recoveries reported earlier.[34]

The repeatability of the separation by 2D UTLC was determined with the reference standard samples (without urine matrix) and with SPE-purified urine samples spiked with benzodiazepines. Urine had only a minimal downward effect on the migration: the distances were 6.7−20.5 mm for the reference standards and 6.2−19.1 mm for the SPE-purified urine samples (Table 2). Standard deviations of all samples were between 0.2 and 1.4 mm and relative standard deviations between 1.2 and 11.7%, indicating good repeatability for the separation (Table 2).

In conclusion, although the 2D UTLC separation with UV densitometric detection provides LODs down to pmol range, the specificity, and in many cases the sensitivity, is not sufficient for trace analysis of benzodiazepines in biological samples. As a next

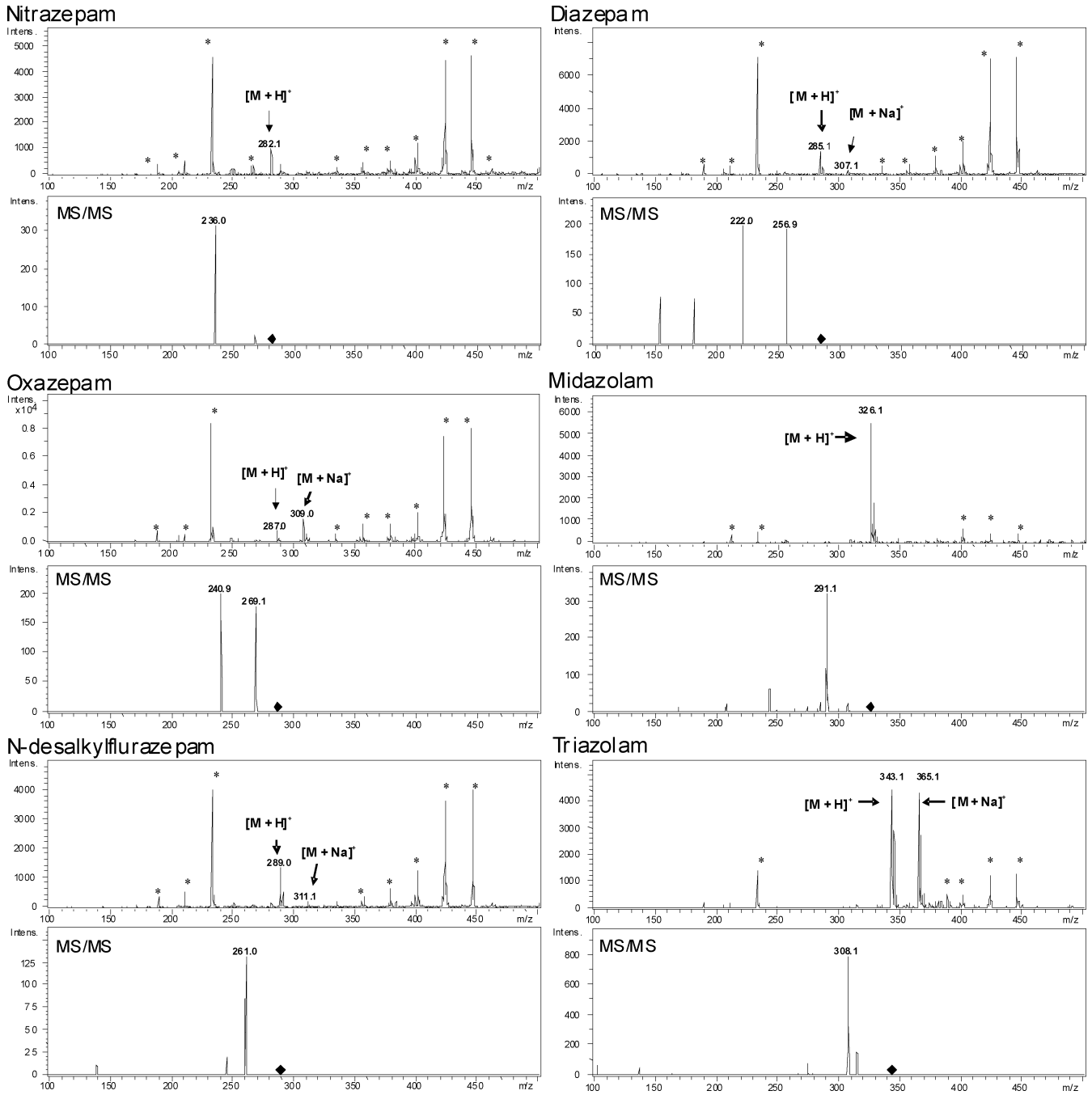

Figure 4. MS and MS/MS spectra of benzodiazepines (spiked in urine) recorded from a 2D developed UTLC plate. The main matrix ions are marked with an asterisk (∗).

step, therefore, we studied the feasibility of AP-MALDI-MS for the detection of benzodiazepines on the UTLC plate. Since the 2D UTLC separation is repeatable, it is sufficient to determine the positions of the zones by dyeing or UV densitometry only once. After that, the AP-MALDI-MS analysis can be done merely by adding the matrix and focusing the laser to the desired place or zone on plate.

AP-MALDI-MS. The selection of the matrix, the amount of matrix applied, and the other instrumental parameters for the AP-MALDI-MS and MS/MS work were based on our previous studies with UTLC and AP-MALDI-MS.[10] The MS and MS/MS spectra of the standard samples were measured by applying 1 nmol of benzodiazepine and 10 nmol of the α-CHCA matrix to the UTLC plate. In MS spectra of the neat α-CHCA matrix (Figure 3A), the most abundant matrix background ions (at m/z 212, 234, 423, 445, and 499) were typically the sodium and/or potassium adducts or dimers of α-CHCA. All MS spectra of the benzodiazepines showed an abundant protonated molecule (Table 3) with minimal fragmentation. Benzodiazepines containing the hydroxyl group (oxazepam and lorazepam) also showed abundant sodium adduct ion. The background disturbance caused by the matrix was relatively low, and no background ions appeared at the same m/z values as the protonated molecules or sodium adducts of the benzodiazepines. These results indicate that 10 nmol of the matrix provides effective ionization of the benzodiazepines, and at the same time being a low enough amount to minimize the disturbance caused

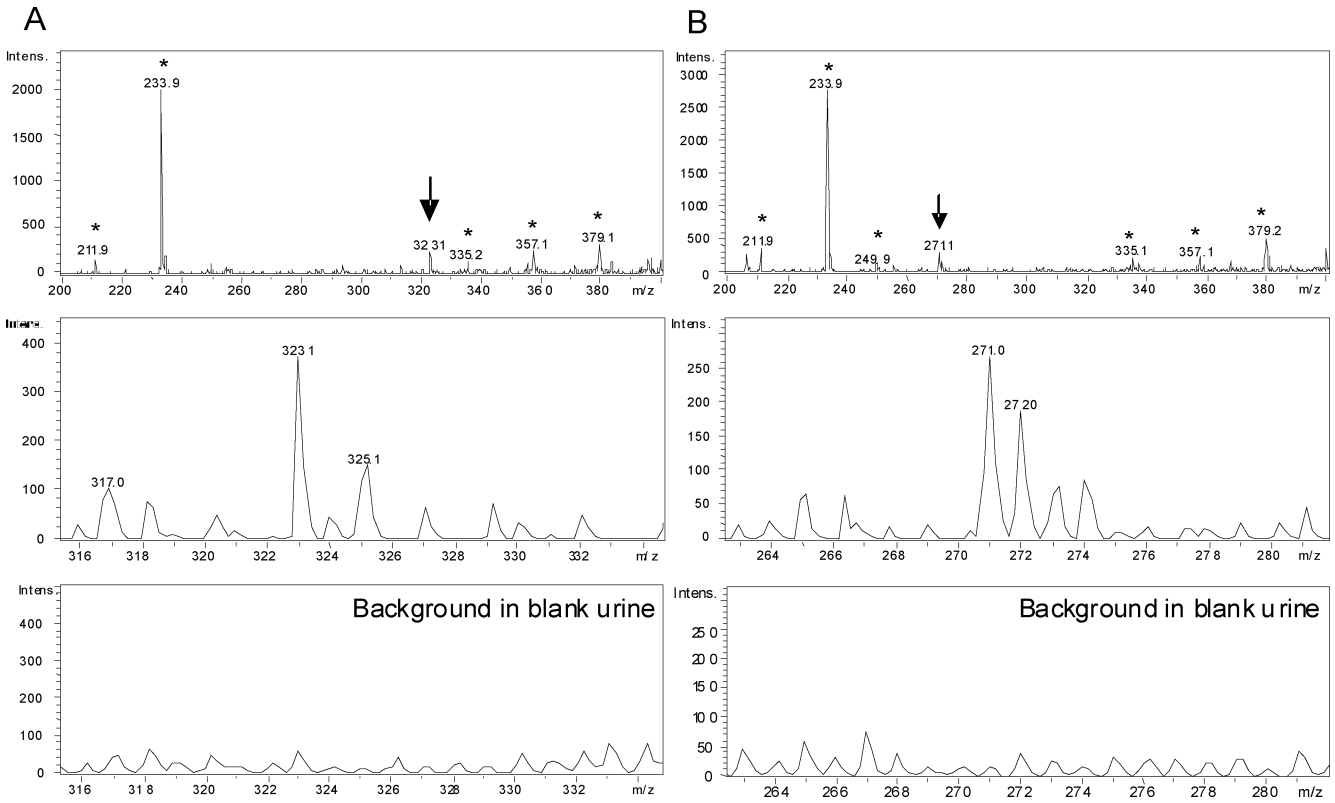

Figure 5. Findings from an authentic urine sample after intake of diazepam. MS spectra were measured from a 2D developed UTLC plate by AP-MALDI-MS. (A) Sodium adduct of temazepam (marked with arrow), and magnification of the chlorine isotope pattern of ion at *m*/*z* 323, and the background in blank urine. (B) Protonated molecule of *N*-desmethyldiazepam (marked with arrow), and magnification of the chlorine isotope pattern of ion at *m*/*z* 271, and the background in blank urine.

by the matrix. In the MS/MS experiments, the product ions (Table 3) were mostly the same as those measured previously by Smyth et al. using an ion-trap instrument.[39]

The AP-MALDI-MS and AP-MALDI-MS/MS spectra were also measured for six SPE-purified urine samples spiked with benzodiazepines (300 pmol) and separated by 2D UTLC. Mostly, the MS spectra (Figure 4) showed the same ions as those measured for the pure reference samples (Table 3), but sodium adducts of the analytes were more abundant in the spiked urine samples than in the standards, despite the SPE purification. Likewise, the MS/MS spectra showed the same product ions in the urine and reference standard samples, indicating that the endogenous compounds in urine do not significantly disturb the analysis of benzodiazepines in MS/MS mode. This result was further investigated by measuring the LODs.

The LODs for benzodiazepines measured by AP-MALDI-MS after UTLC separation were 2.0−66.7 pmol in pure solvent and 7.9−97.0 pmol in SPE-purified urine (Table 1). Thus, the endogenous compounds in urine do in fact disturb the analysis in MS mode, as LODs were 2−10 times higher in urine. The disturbing endogenous compounds do not suppress the ionization, however, since the LODs measured by MS/MS were mostly at the same level for benzodiazepines in pure solvent and in SPE-purified urine. The LODs for MS/MS were mostly below 10 pmol, and about 10 times lower than that for MS (Table 1).

The effect of the Dragendorff reagent (used for visualization of the benzodiazepines as derivatives) on the MALDI-MS spectra of benzodiazepines was investigated as above but after spraying of the plate with the Dragendorff reagent. This reagent contains potassium iodide, and therefore the MS spectrum of the neat α-CHCA matrix shows potassium adducts and dimers of the matrix (*m*/*z* 228, 266, 417, 455, 477, and 493) (Figure 3B). However, the MS spectra of the benzodiazepine derivatives measured after spraying showed abundant protonated molecule (just as without the reagent), although their absolute abundances were about 10−100 less with than without the reagent. Also the background disturbances caused by the matrix and Dragendorff reagent were increased. The results show that the Dragendorff reagent suppresses the ionization of the benzodiazepines and should be used only where concentrations of the analytes are relatively high (100−500 pmol).

Qualitative Analysis of Authentic Urine Sample. The 2D UTLC-AP-MALDI-MS method was applied in the qualitative analysis screening of an authentic urine sample after intake of a single dose of 10 mg of diazepam (Diapam). Since diazepam has a rather long (about 20−100 h) half-life, the urine was collected 53 h after the intake. Diazepam is mainly metabolized to *N*-desmethyldiazepam (MW = 270), temazepam (MW = 300), and oxazepam (MW = 286) and their glucuronides. The metabolites, too, have long half-lives: temazepam and oxazepam about 15−20 h and *N*-desmethyldiazepam as much as 30−200 h. First, 20 μL of enzymatic hydrolyzed and SPE-purified authentic urine sample was applied to the UTLC plate. After 2D UTLC separation, MALDI matrix was added to the plate at the known positions of the separated zones. Because the 2D UTLC separation was highly repeatable and the width of the separated benzodiazepine zones

(39) Smyth, W. F.; McClean, S.; Ramachandran, V. N. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2061−2069.

on UTLC plate was about 2−4 mm (in x and y dimensions), and wider than the standard variations of the migration distances (0.2−1.4 mm), the addition of the matrix and focusing of the laser to the right locations in the AP-MALDI-MS analysis can be done without prior detection of the zones by dyeing or UV densitometry. Two possible metabolites of diazepam were detected in the authentic urine sample by 2D UTLC-AP-MALDI-MS. The MS spectra in Figure 5 show ions at m/z 323 (Figure 5A) and 271 (Figure 5B) along with the unmistakable isotope peak of chlorine: Neither ion is seen in the MS spectra of blank urine. The ions correspond to the sodium adduct of temazepam and the protonated molecule of *N*-desmethyldiazepam, respectively. These are the expected findings after intake of diazepam.

CONCLUSIONS

The suitability of 2D UTLC-AP-MALDI-MS for difficult bioanalysis has been demonstrated for the first time. The feasibility of the method was tested in the determination of benzodiazepine spiked in urine. The relative standard deviation of the position of the zones on the UTLC plate was below 10%, indicating good repeatability of the separation. The limit of detection down to pmol range provided detection of benzodiazepine metabolites in an authentic urine sample. This level of sensitivity in bioanalysis can be achieved with the use of UTLC plates; as shown in our earlier work,[10] the sensitivity with HPTLC plates is about 10−100 times worse. The 2D UTLC-AP-MALDI-MS combination provided an easy and as rapid method as LC-MS for the qualitative analysis of an authentic urine sample. The method could also be valuable in metabolomics and in the identification of peptides in proteomics.

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