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Determination of 1,4-benzodiazepines and their metabolites by capillary electrophoresis and high-performance liquid chromatography using ultraviolet and electrospray ionisation mass spectrometry

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Abstract

A study is presented for the separation and determination of fifteen 1,4-benzodiazepine drugs and metabolites by capillary electrophoresis (CE) compared with high-performance liquid chromatography (HPLC). A comparison is made between the CE determination of the compounds by conventional UV detection and LC determination with electrospray ionisation (ESI) ion-trap mass spectrometry. CE is shown to provide superior separation to HPLC but the MS–MS capability of the ion-trap allows for the specific detection and determination of four of the compounds, diazepam, *N′*-desmethyldiazepam, oxazepam and temazepam in the hair of a patient under clinical treatment with diazepam and temazepam. Selected mixtures of drugs and metabolites are determined by CE and LC and the determination of diazepam and its metabolites by CE–UV–ESI-MS–MS is also presented.

Keywords: Sample stacking; Hair; Benzodiazepines; Diazepam; Oxazepam; Desmethyldiazepam; Temazepam

1. Introduction

The 1,4-benzodiazepines are known primarily for their hypnotic and sedative effects, but are also used as anxiolytics, anticonvulsants and muscle relaxants. Their biopharmacological activity has been investigated [1] and comprehensive studies of their chemistry have been made [2,3]. The mood-altering properties of these compounds combined with their ability to stave off the effects of opiate withdrawal have encouraged their availability on the illicit drugs market. On the ''rave'' scene they are known as ''downers'' because they reduce the after effects of LSD, amphetamine and Ecstasy. Recently the capsule form of temazepam has been withdrawn in the UK [4].

A variety of methods exist in the literature for the detection and determination of 1,4-benzodiazepines in biological matrices. Gas chromatography–mass spectrometry (GC–MS) determinations have been reviewed by Maurer [5], further studies have also been carried out [6–8] and comparisons made between this technique and various immunoassays [9,10]. High-performance liquid chromatography (HPLC) methods have been studied [11,12] and a critical evaluation made of the application of capillary electrophoresis (CE) to the detection and determination of 1,4-benzodiazepines in formulations and body materials [13].

In recent years the coupling of LC to MS has

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provided a useful and rugged technique for the analysis of drug compounds and an alternative to GC–MS in which some compounds thermally decompose to give a metabolite common to many of the benzodiazepines [14,15]. The advent and development of soft-ionisation techniques such as electrospray ionisation (ESI) [16,17], ion-trap mass spectrometry which allows MS^n characterisation of compounds [18] and the coupling of the two [19,20] has opened further avenues for the specific detection and determination of various pharmaceuticals. Method development is an important factor as involatile sodium salts, phosphates, borates or nitrates, commonly used in HPLC are not compatible with the electrospray process due to adduct formation and precipitation in the mass spectrometer.

The characterisation of chlordiazepoxide and an unknown benzodiazepine was carried out by successively capturing and fragmenting their product ions (MS^n experiments) using ion-trap MS and confirmed that the unknown was an analogue of chlordiazepoxide [21]. Such fragmentation builds up a fingerprint of each compound under investigation and may then be used for the specific identification of the analyte in a complex mixture. The determination of diazepam, *N′*-desmethyldiazepam, nitrazepam, flunitrazepam and medazepam in human serum and urine has been reported by HPLC–ESI-MS–MS capable of quantifying 2 ng ml^{-1} levels in serum [22].

While LC–MS is now a fairly well established technique, CE–MS has not quite reached that status due to problems associated with the low electrolyte flow exiting the capillary and entering the ESI-MS interface. This presents difficulties in maintaining a stable flow of ions into the mass spectrometer and as a result much work has centred upon developing a suitable ESI interface [23–25]. A review of applications of CE–MS has been produced and concluded that the popularity of this technique will grow as more sensitive MS instrumentation and the CE–MS interface are developed [26]. An early application worthy of note is that for the determination of flurazepam and *N*-(1-hydroxyethyl)flurazepam by CE–UV–MS–MS. The volatile salt ammonium acetate prepared in 15–20% methanol was used as run buffer and a limit of detection (LOD) of 0.5 μg ml^{-1} for *N*-(1-hydroxyethyl)flurazepam in urine was established [27].

The analysis of drugs in hair is very topical, has been widely studied [28,29] and is also the subject of recent LC–ESI-MS analysis [30]. While it is increasing in popularity, attention must be paid to the ever-present controversy surrounding decontamination of the hair surface to distinguish between exogenous and endogenous components [31]. Too mild a treatment procedure will result in the possibility of false positives while too harsh a wash procedure may result in endogenous compounds leeching from within the matrix. Leaving this debate aside, however, the attractiveness of the technique is beyond question as far as building up a time-history of abuse of a controlled substance is concerned.

Following decontamination of the surface, a wide variety of extraction procedures have been employed for other drug compounds. These include acid or base treatment followed by solvent extraction [32], sonication in solvent at elevated temperature [33] and pulverisation of the hair sample followed by liquid–liquid extraction and solid-phase extraction for further purification [34]. A wide variety of other extraction procedures are detailed by Kintz [29].

Various extraction methods for 1,4-benzodiazepines from hair and decontamination procedures used have been studied [35–42].

The ideal scenario is to keep the drug compound intact throughout the extraction procedure. For that purpose automated Soxhlet apparatus is used in this paper for the extraction of the 1,4-benzodiazepines from hair. To date this application has not appeared in the literature, though a modification of the Soxhlet apparatus has been reported for the extraction of phenobarbital and amitriptyline from biological fluids [43].

This paper is concerned with the development of CE–UV and LC–ESI-MS^n (where $n=1$ or 2) methods for the determination of 15 1,4-benzodiazepines, both as complex mixtures and in groups of parent compound with available metabolites. The LC–ESI-MS^n method is applied to the determination of diazepam and its metabolites in hair following Soxhlet extraction of the drugs from the hair matrix. A CE–UV–MS^n method is also presented for the determination of diazepam and its metabolites, though the methodology lacks the sensitivity to determine these compounds in hair, except, perhaps in overdose cases. Large volume sample stacking (LVSS)-CE of drugs has been carried out [44] and

has been seen to significantly lower the LOD. However, the necessary presence of molecules such as cetyltrimethylammonium bromide (CTAB) in the run electrolyte causes signal suppression in CE–ESI-MS [45]. For that reason LVSS-CE–ESI-MS was not attempted in these studies.

2. Experimental

2.1. Instrumentation

2.1.1. UV spectrometry

UV spectrometry of the drugs was performed with a Phillips PU 8730 fixed-bandwidth spectrometer, Phillips Scientific (Cambridge, UK).

2.1.2. Capillary electrophoresis

Conventional CE investigations with UV and MS detection were carried out using a CE Ultra instrument from ThermoQuest (San Jose, CA, USA). For UV detection studies separations were achieved using an untreated fused-silica capillary of 70 cm× 75 μm I.D. (Composite Metal Services Hallow, UK). A window was burned in the polyimide coating at 58 cm, detection taking place by means of a UV3000 UV–Vis fast-scanning detector (ThermoQuest). MS^n characterisation and detection of the drug compounds took place using an LCQ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) utilising ESI. For MS determinations the capillary length from injection point to UV detection window was 34 cm after which the capillary travelled a further 61 cm to its point of entry into the mass spectrometric interface at the end of the electrospray needle. Peak area integrations were calculated using PC 1000 software.

LVSS investigations were performed with a Spectrophoresis 1000 instrument manufactured by ThermoSeparation Products (Manchester, UK). The capillary dimensions and characteristics were similar to those used in the conventional CE studies as detailed above.

2.1.3. High-performance liquid chromatography

The LC system used in these studies was supplied by ThermoQuest and comprised a P4000 pump, AS 3000 autosampler, 20-μl injector loop, on-line UV 1000 UV detector and an SCM 1000 vacuum membrane degasser. The column used for chromatography separations was a Phenomenex (Macclesfield, UK) Luna C_{18} column; 15 cm×4.6 mm I.D. A guard column of similar characteristics but 30 mm in length was positioned just before the analytical column. For mass spectrometric studies the LC system was interfaced with the LCQ instrument.

2.1.4. Soxhlet extraction of drugs from hair samples

For the extraction of drug compounds from the hair matrix, automated Soxhlet extraction was performed using a Tecator Soxtec System HT2 with a 1046 Soxtec Service unit (Tector, Höganäs, Sweden). 24.5-mm internal diameter thimbles were used to contain the hair samples and HPLC-grade methanol used for the extractions.

2.2. Reagents

All solvents were of HPLC-grade while other chemicals used were of analytical-reagent quality. Methanol, acetonitrile, acetic acid, boric acid and sodium hydroxide were obtained from BDH (Poole, UK). Citric acid and CTAB were obtained from Aldrich (Poole, UK). Disodium tetraborate (Borax) was obtained from Hopkin and Williams (Chadwell Heath, UK). Temazepam, oxazepam and lorazepam were bought from Sigma, while all the other drug compounds were obtained from Roche Products (Welwyn Garden City, UK). Milli-Q 18 MΩ water (Millipore) was used throughout. Standards of the drugs were prepared by dissolving an appropriate mass in 25 ml of methanol to provide a concentration of $1.0 \cdot 10^{-3}$ mol l^{-1}. Nitrogen gas for the LCQ instrument delivered from a Whatman nitrogen generator (Whatman, Haverhill, MA, USA) while the helium damping gas, present in the ion-trap was obtained from BOC Medical Gases (Guildford, UK).

2.3. Procedures

2.3.1. UV study of the compounds

In order to determine suitable absorption wavelengths of the parent drug compounds for UV monitoring purposes, 1-cm path-length vials were filled with 2.0 ml of Milli-Q 18 MΩ water and 50 μl of a 10^{-3} mol l^{-1} methanolic stock of the drug. The reference vial contained 2.0 ml of Milli-Q 18 MΩ

water and 50 μl of methanol. Scans were initiated at periodic intervals during a 20-min time period over the wavelength range 200–400 nm and the resulting profile recorded.

2.3.2. Determination of pK_a values by UV spectrometry

UV spectrometry was used to determine pK_a values for three of the compounds; *N′*-desmethyldiazepam, flunitrazepam and 3-hydroxyflunitrazepam. A stock solution of Britton Robinson buffer consisting of 0.04 mol l^{-1} acetic, phosphoric and boric acids was pH adjusted with 0.2 mol l^{-1} NaOH to cover the pH range from 1.00 to 4.50 in 0.25 intervals. For the blank reference cell 3.0 ml of Britton Robinson buffer and 250 μl of methanol were used. The sample cell was filled with 3.0 ml of buffer and $1.0 \cdot 10^{-3}$ mol l^{-1} drug, and a scan initiated immediately in the range 210 to 300 nm. The wavelength of maximum absorbance was noted and the data plotted graphically in the form absorbance vs. pH and the resulting pK_a value calculated.

2.3.3. Conventional CE

An initial start-of-day wash procedure was used to condition and equilibrate the capillary. This consisted of a 0.4-min wash with 0.1 mol l^{-1} NaOH at 60°C after which the capillary was flushed with Milli-Q water, also at 60°C for 0.4 min. The final step was to rinse the capillary with run buffer at 30°C for 0.8 min under a pressure of $6.895 \cdot 10^{-1}$ MPa. Conditioning then took place by applying the run potential to the buffer-filled capillary until a constant run current was achieved. (Short wash times are used due to the pressure injection system that operates on the CE Ultra instrument and are equivalent to the wash times employed for the CE 1000 instrument as detailed later).

The run buffer consisted of 0.02 mol l^{-1} citric acid with 15% methanol as modifier, the pH of the solution being 2.50. A separation potential of 20 kV was used, the temperature maintained at 30°C and the detector set at 230 nm. Samples were prepared in Milli-Q water by serially diluting $1.0 \cdot 10^{-3}$ mol l^{-1} methanolic stock solutions of the drugs, and along with buffers and wash solutions they were filtered using 13-mm diameter discs with pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI, USA) prior to analysis. The capillary was washed with run buffer prior to each pressure injection at $5.516 \cdot 10^{-3}$ MPa for 2.5 s.

Initially a mixture of all 15 available compounds was prepared in water at a concentration of $4.0 \cdot 10^{-5}$ mol l^{-1}. Following this the compounds were analysed in their various ''family'' groups of parent compound plus available metabolites, a scenario which would be of particular interest in clinical or forensic situations. Under these conditions calibration data was obtained for the drugs over the ranges shown in Table 3. The lowest concentration used in each case does not refer to the limit of quantitation, but to an arbitrarily chosen value. The LOD (being defined as the concentration that gave a response greater than the blank by three-times the standard deviation of its value) was calculated for each drug and metabolite. Relative standard deviation (RSD) values were calculated at the $2.5 \cdot 10^{-5}$ mol l^{-1} level on both migration time and peak area where $n=6$ in all cases.

2.3.4. Large volume sample stacking capillary electrophoresis

As above an initial start-of-day procedure was used to thoroughly wash and condition the capillary. The same constituents are used, however the vacuum system on the CE 1000 requires that the wash times be increased to 5 min. Sample was introduced into the capillary by hydrodynamic injections of up to 30 s in duration. For the purpose of LVSS-CE a number of null volume injections were made in which only run buffer was present in the capillary in order to determine the average run current under the applied potential. Following injection of the sample a potential of +12.5 kV was applied to remove the solvent from the sample plug and to stack the compounds. The run current was allowed to reach 95% of its pre-injection value before the separation potential of −12.5 kV was applied. The temperature was maintained at 20°C and the detector set to 230 nm throughout. The buffer used for these investigations was 0.05 mol l^{-1} borax and 0.002 mol l^{-1} CTAB, adjusted to pH 2.20 with orthophosphoric

acid. These conditions were found to be acceptable to stacking of the compounds.

2.3.5. Capillary electrophoresis–mass spectrometry

For these studies the CE Ultra instrument was interfaced with the LCQ mass spectrometer. This procedure involves installing a needle and nozzle of greater internal diameter on the ESI probe than is used for LC–MS in order to accommodate the greater thickness of capillary used in CE. Due to the low flow-rate from the CE, it is necessary to use a sheath liquid to provide a make-up flow in the interface to assist with ionisation and also to complete the circuit thus allowing electrophoresis to take place. In these studies the sheath liquid consisted of methanol–water–acetic acid (50:50:1); and was filtered and sonicated for 20 min before infusion into the LCQ instrument at a rate of 2.0 μl min^{-1}. Spray voltage was set to 4.5 kV, capillary temperature to 200°C; the sheath gas flow was maintained at 20 units (arbitrary value set by the software) while the auxiliary gas was not used. By operation of a micrometer installed on the front of the ESI probe it is possible to adjust the position of the capillary end relative to the nozzle tip. This is necessary for achieving a stable spray current before measurements are made. Typically current values of around 1–2 μA are acceptable for background spray current under these conditions.

A 75-μm capillary was used for the separations but as it must now protrude from the CE instrument and reach the MS system, its length is increased to 95 cm. Conditioning of the capillary took place as follows; 1.0 mol l^{-1} NaOH, 0.1 mol l^{-1} NaOH and water are washed through the capillary at 60°C for 1 min under a pressure of $6.895 \cdot 10^{-1}$ MPa. The washes are carried out in duplicate so that the part of the capillary positioned outside the oven is still purged with solution at elevated temperature. The various solutions were allowed to stand for at least 5 min in the capillary before the next stage of the wash procedure took place. It is important that these washes are made with the ESI probe retracted from the mass spectrometer to avoid NaOH entering the source. Conditioning of the capillary then took place by flushing the capillary with run buffer which consisted of 0.02 mol l^{-1} citric acid and 15% methanol. Injection of sample took place by applying a pressure of $1.103 \cdot 10^{-2}$ MPa to the sample vial for 1.25 s. A potential of 25 kV was applied to the leading electrode on the CE apparatus, providing an overall separation potential of 20.5 kV.

2.3.6. LC–MS investigations

For the determination of a mixture of all 15 available compounds, a stock solution of them was prepared in methanol at a concentration of $5.0 \cdot 10^{-5}$ mol l^{-1}. Tertiary elution was used for the determination of this mix, in which the solvent used was Milli-Q water–acetonitrile–methanol (30:5:65) over a run time of 25 min at a flow-rate of 0.25 ml min^{-1}. Twenty-μl injections were made via the autosampler.

For the determination of individual compounds and their metabolites a simpler isocratic system with a mobile phase consisting of acetonitrile–water (65:35) was used throughout. For diazepam and its metabolites this provided a slightly better efficiency of separation. However, the need for perfect separations is not necessary due to the sensitivity and resolution power of the LCQ instrument.

MS and MS–MS characterisations were carried out using an LCQ ion-trap mass spectrometer. In order to optimise the ionisation of the molecules parameters such as the auxiliary and sheath gas flows the capillary temperature and the collision energy were altered until the maximum possible signal for a given concentration of drug was obtained. In these studies diazepam, and its three major metabolites, oxazepam, temazepam, and *N′*-desmethyldiazepam were of greatest interest. The instrument was therefore tuned to provide the best possible signals for these species and these parameters then kept constant for all the other compounds.

Apart from the diazepam family of compounds the collision energy for MS–MS characterisations was kept at the instrument default value of 25% throughout. This value was also used for diazepam itself, but lowered to 20% for oxazepam, nordiazepam and temazepam. These values refer to the percentage of available energy applied to the resonating parent ions to increase the number of collisions with helium molecules in the ion-trap. The higher the energy that is applied, the greater the fragmentation that will occur. The sheath gas flow was set to 60 (arbitrary unit defined by the software) and the auxiliary gas to 30. The capillary temperature was set to 220°C and

the spray voltage to 3.5 kV. These values were then kept constant for the other groups of compounds.

2.3.6.1. Calculation of LODs

The major parent compounds and their various metabolites had their LODs, RSD values and peak efficiency calculated. All the analyses were carried out using the LCQ quantitation software in which two criteria of retention time and MS–MS characteristic must be satisfied before a peak is identified. This therefore increases the selectivity of the techniques immensely, and by the use of scan filters it is possible to accurately determine co-eluting species in a single analysis as the filter is effectively a separate detection device unique to each compound. Using these parameters it is possible to identify components of a mixture with extreme accuracy, and this may be made even more specific by employing MS^3 characterisation of the species perhaps in conjunction with continuous reaction monitoring (CRM) of the product ions formed; as defined by the LCQ software. The amount of analyte present is calculated by comparison with a previously constructed calibration graph.

LOD determinations are usually made with reference to analysis of blank signals, and the LOD then defined as a concentration that gives a signal significantly greater in magnitude than that of the blank by some predetermined factor. However, in MS–MS determinations the conventional background does not theoretically exist as only peaks due to a specific daughter ion from a parent compound of specified mass are ever recorded. The likelihood of a contaminant in the blank exhibiting those identical characteristics is very small indeed, if not virtually impossible. Under such circumstances LODs may therefore only be made by setting instrument integration parameters to detect peaks above a signal-to-noise (S/N) ratio of 2 or 3 and then serially diluting standards of compounds under investigation until the signal completely disappears. The LOD would then be taken as the last concentration that caused a peak to be detected.

In our studies it was found that after a typical analysis consisting of standards, blanks and unknowns, some of the compounds would remain in the system for perhaps days after the initial analyses. This was also the case after methanol washing of the columns, injectors and ESI interface in accordance with procedures recommended by the instrument manufacturer. This resulted in some of the compounds being detected in blank methanol, and therefore forced us to return to a conventional definition and calculation of LOD under these circumstances. A number of blank samples were run consisting of methanol only and the mean response for each compound noted. The LOD was then defined as the concentration that gave a signal twice that blank response.

2.3.7. Hair extraction procedure

A sample of hair weighing 149.00 mg was obtained from a patient being treated with 8 mg diazepam and 20 mg temazepam per day though the exact duration of the treatment was not known. The sample was washed to remove exogenous compounds by five consecutive washes with 5 ml of methanol. After being dried the sample was cut into 1 mm lengths (approx.), weighed and 124.81 mg transferred to a thimble compatible with the Soxhlet extraction system. Fifty ml of methanol was used for the extraction and the temperature control set to 160°C. Tap water was supplied to the condenser at an approximate rate to 2 l min^{-1}. The sample was boiled for 3 h with the thimble in contact with the solvent, after which the thimble was raised to the rinsing position for 2 h during which time the solvent dropped through the sample from the condenser. After the extraction process the solvent was evaporated, the residue filtered and the sample made up to a total of 1.0 ml in methanol. The samples were then subjected to LC–MS analysis, 20 μl being injected each time. A fuller description of the decontamination procedure and its effectiveness is provided in a future publication [46].

3. Results

3.1. UV study of the compounds

In neutral media, such as the LC mobile phase, 230 nm was a suitable wavelength common to most of the compounds, and was therefore chosen for UV detection studies.

3.2. Determination of pKa values by UV spectrometry

On plotting the data achieved a point of inflexion was noted for each of the three compounds investigated. Their calculated values were; *N′*-desmethyldiazepam, 3.50; flunitrazepam, 1.38 and 3-hydroxyflunitrazepam, 1.70. They together with other pK_a values are shown in the Table 1. The *N′*-desmethyldiazepam pK_a was recalculated to confirm the literature value quoted. These values were used to help explain the elution order of the compounds in mixtures by conventional CE.

3.3. Conventional CE

The CE Ultra instrument was used for the determination of mixtures of the 1,4-benzodiazepines compounds. Initially a mixture of all 15 available compounds was prepared in water at a concentration of $4.0 \cdot 10^{-5}$ mol l^{-1}. This resulted in resolution of 12 of the compounds as shown in Fig. 1a compared with the LC–MS separation of the same mix in Fig. 1b.

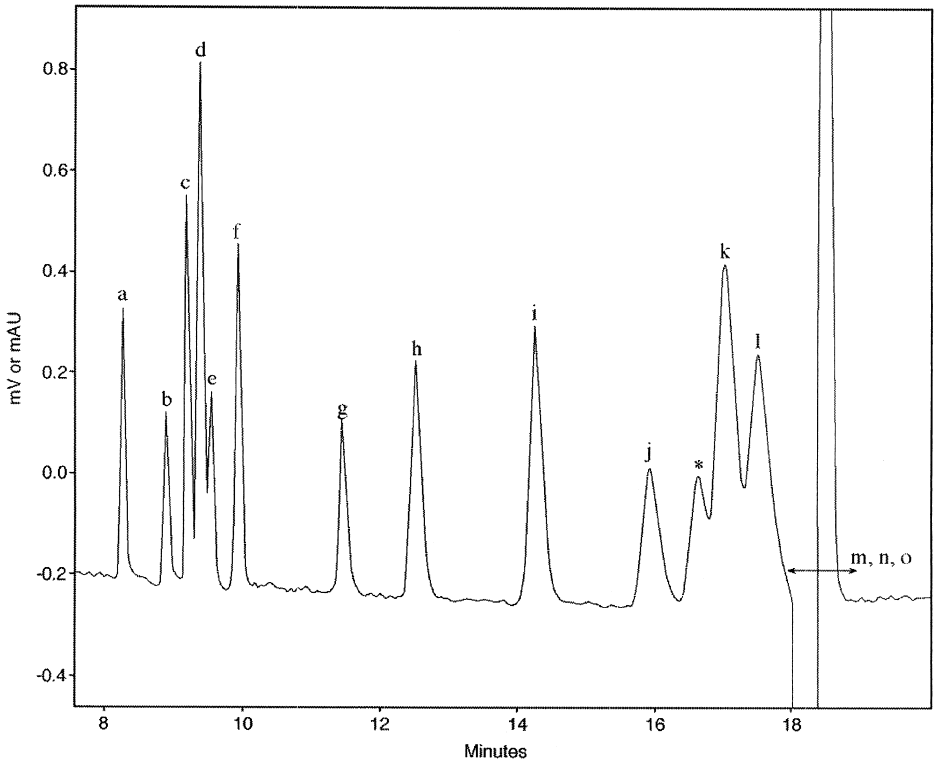
As expected 7-aminonitrazepam which is protonatable on the $-NH_2$ group (pK_a 4.6) and the C=N group (pK_a 2.5) migrates first through the capillary. It is followed by singly charged *N′*-desmethyldiazepam which migrates ahead of singly charged chlordiazepoxide possibly due to differences in there respective hydrodynamic radii. Flurazepam is another anomaly in that at pH 2.5 it is expected to have a charge $>+1$ and to migrate just behind 7-aminonitrazepam. This may also be explained by differences in hydrodynamic radii. On the whole the remainder of the species were separated in line with their pK_a values corresponding to the monoprotonated species. The last three species' migration is complicated by the migration of a vacancy which is present in the blank. This is a phenomenon encountered in CE when high or low pH buffers are used.

According to Yang and Lu [50] protonation of the chlordiazepoxide molecule occurs at the nitrogen of an imine bond between C2 carbon and its nitrogen substituent, rather than at the *N*-oxide as previously thought. This will therefore explain the relatively long migration time for the chlordiazepoxide metabolite demoxepam which is unable to undergo protonation in the same way and therefore remains

Table 1
pK_a data for the 15 1,4-benzodiazepines investigated in these studies (the annotation provided refers to the peak designations in Fig. 1a and b)

Compound	pK_a value	Annotation of peak in Fig. 1a and b
7-Aminonitrazepam[a]	2.5, 4.6, 13.1	A
N-Desmethyldiazepam[a]	3.5, 12.0	B
Chlordiazepoxide[b]	4.6,	C
Flurazepam	1.42, 8.1	D
7-Acetamidonitrazepam[a]	3.2, 12.4	E
Diazepam[b]	3.3	F
Nitrazepam[b]	3.2, 10.8	G
N′-Desalkylflurazepam	2.57	H
N′-(2-Hydroxyethyl)flurazepam	2.26	I
3-Hydroxyflunitrazepam	1.7	J
Oxazepam[b]	1.7	K
Temazepam[c]	1.6	L
Lorazepam[c]	1.3, 11.5	M
Flunitrazepam	1.4	N
Demoxepam[d]	10.7	O

[a] Ref. [47].
[b] Ref. [48].
[c] Ref. [49].
[d] Ref. [50].


mV or mAU
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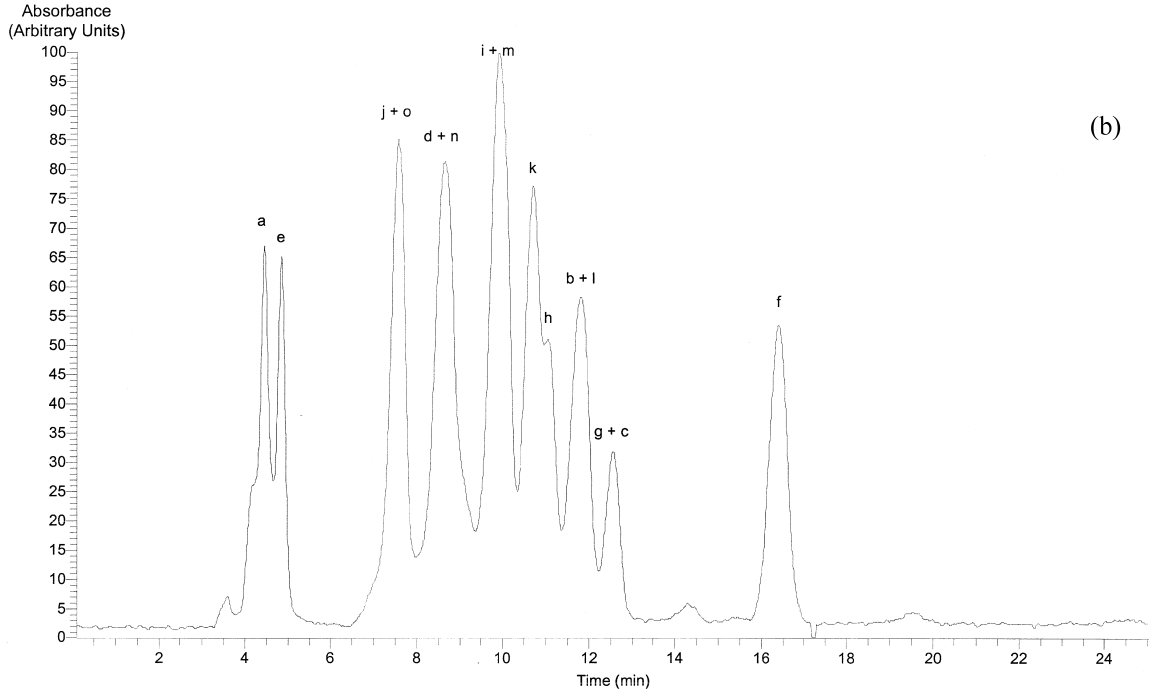
(a)


Absorbance
(Arbitrary Units)
Time (min)
a
e
j + o
d + n
i + m
k
h
b + l
g + c
f

(b)

Table 2
LOD and RSD data for 15 1,4-benzodiazepines by conventional CE

Compound	LOD (mol l^{-1})	Migration time (min)	RSD of migration time (%)[a]	RSD of peak area (%)[a]
Diazepam	$5.36\cdot10^{-6}$	10.112	0.48	12.06
Oxazepam	$1.28\cdot10^{-6}$	17.404	0.85	13.74
Temazepam	$2.02\cdot10^{-6}$	17.900	0.92	11.76
N′-Desmethyldiazepam	$1.55\cdot10^{-6}$	9.069	0.46	12.13
Chlordiazepoxide	$2.88\cdot10^{-7}$	9.000	0.62	10.43
Demoxepam	$5.00\cdot10^{-5}$	16.655	0.63	5.07
Nitrazepam	$5.12\cdot10^{-7}$	11.895	10.37	17.89
7-Acetamidonitrazepam	$5.63\cdot10^{-7}$	9.750	7.47	20.04
7-Aminonitrazepam	$7.8\cdot10^{-8}$	8.400	5.59	13.15
Flurazepam	$3.89\cdot10^{-7}$	9.105	0.67	13.13
N′-(2-Hydroxyethyl)flurazepam	$1.43\cdot10^{-6}$	13.600	0.89	14.36
N′-Desalkylflurazepam	$1.28\cdot10^{-6}$	11.986	0.83	8.14
Flunitrazepam	$5.67\cdot10^{-6}$	22.063	1.92	12.26
3-Hydroxyflunitrazepam	$6.45\cdot10^{-7}$	20.814	1.84	11.38
Lorazepam	$4.00\cdot10^{-5}$	11.435	12.77	15.57

[a] The RSD values were determined at the $2.5\cdot10^{-5}$ mol l^{-1} level for each compound.

uncharged. The absence of electroosmotic flow at pH 2.50 will also contribute to the long migration time for this molecule.

Each family group was then taken it turn and the separation behaviour of each examined. LODs and RSD values are given in Table 2 while calibration data is detailed in Table 3. In most cases the calibration range was $1.0\cdot10^{-4}$ to $2.5\cdot10^{-6}$ mol l^{-1}. However, due to lorazepam and demoxepam migrating with a vacancy they are only ever observed at concentrations greater than $4.0\cdot10^{-5}$ mol l^{-1}. For each family group of compounds baseline resolution is achieved for parent compound and metabolites. The only exception is the diazepam case where fused peaks are seen for oxazepam and temazepam but they are still sufficiently resolved for easy identification.

3.4. LVSS-CE

Using 0.05 mol l^{-1} borax buffer containing 0.002 mol l^{-1} CTAB adjusted to pH 2.2 with orthophosphoric acid, the following results were obtained when a 1×30 s hydrodynamic injection of the compound in water only was made. Under the influence of a +12.5 kV potential, the solvent took 8.7 min to pump out. Analysis then took place of various drug compounds either separately or in mixtures, at relatively high concentration levels typically $1.0\cdot10^{-4}$ mol l^{-1} to ensure that a good analytical signal was produced in these initial studies.

While CE is notorious for shifting migration times the problem seems much exaggerated when LVSS is used, particularly in the case of mixtures of the benzodiazepines. When analysed singly diazepam had a migration time of 38.03 min under these analytical conditions. In LVSS-CE migration times are longer than for conventional CE as separation takes place in reverse-polarity mode. In a mixture with flurazepam the migration time for diazepam shifted to 39.95 min, flurazepam migrating earlier with a time of 35.95 min. Addition of nitrazepam to this mix results in further complexity with only two peaks being seen in the profile. This may be due to co-migration of two of the species as a relatively

Fig. 1. (a) Separation of a $4.0\cdot10^{-5}$ mol l^{-1} mix of 15 1,4-benzodiazepines by CE–UV. The run electrolyte was 0.02 mol l^{-1} citric acid and 15% methanol. A separation potential of +20 kV was used. An asterisk (*) refers to an unidentified species. (b) Determination of the same mixture by LC–MS. Tertiary elution was used for the determination of this mix, in which the solvents used were Milli-Q water–acetonitrile–methanol (30:5:65) over a run time of 25 min at a flow-rate of 0.25 ml min^{-1}. Twenty μl injections were made via the autosampler. The annotation of the peaks in both cases is as shown in Table 1.

Table 3
Calibration data for 15 1,4-benzodiazepines by conventional CE

Compound	Correlation coefficient (R^2)	Range (mol l^{-1})	n
Diazepam	0.9971	$2.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	8
Oxazepam	0.9942	$2.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	8
Temazepam	0.9940	$2.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	8
N′-Desmethyldiazepam	0.9835	$2.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	8
Chlordiazepoxide	0.9977	$2.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	7
Demoxepam	0.9974	$5.0\cdot10^{-5}$ to $1.0\cdot10^{-4}$	4
Nitrazepam	0.9979	$2.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	7
7-Acetamidonitrazepam	0.9942	$2.5\cdot10^{-6}$ to $7.5\cdot10^{-5}$	7
7-Aminonitrazepam	0.9882	$2.9\cdot10^{-6}$ to $8.8\cdot10^{-5}$	7
Flurazepam	0.9896	$7.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	6
N′-(2-Hydroxyethyl)flurazepam	0.9936	$1.0\cdot10^{-5}$ to $1.0\cdot10^{-4}$	5
N′-Desalkylflurazepam	0.9986	$7.5\cdot10^{-6}$ to $1.0\cdot10^{-4}$	6
Flunitrazepam	0.9970	$7.5\cdot10^{-5}$ to $1.0\cdot10^{-4}$	6
3-Hydroxyflunitrazepam	0.9898	$7.5\cdot10^{-5}$ to $1.0\cdot10^{-4}$	6
Lorazepam	0.9869	$4.0\cdot10^{-5}$ to $1.0\cdot10^{-4}$	4

large peak is observed together with a smaller one, however the migration times shifted significantly to 32.48 and 35.93 min for flurazepam and diazepam, respectively. Analysed individually nitrazepam has an observed migration time of 39.63 min suggesting that perhaps some form of preferential stacking takes place when mixtures are analysed in this way.

Migration time data and efficiency values (N= plates calculated by the half-width method) for other compounds are given in Table 4.

As in accordance with other studies [44] run buffer was added to the sample at a level of 10% in order to increase conductance within the capillary. This was initially successful in allowing the current to reach its usual level, and as expected, in a much faster time than previously achieved. Typical pumping out times for the samples are as follows; 1×30 s injection, 0.92 min; 2×30 s injections, 2.75 min.

In the case of the sample containing 1% run buffer, a suitable trade-off is achieved between solvent removal and stacking efficiency. The conductivity in the capillary is lower than in the 10% case so the stacking process takes a slightly longer time. However, the lower concentration of run buffer

Table 4
Efficiency values and migration time data for 1,4-benzodiazepines by LVSS-CE[a]

Compound	Migration time (min)	Peak efficiency
Chlordiazepoxide	34.42	64 301
Diazepam	38.03	54 512
Nitrazepam	39.63	13 638
Flurazepam	35.95[b]	70 145
Flunitrazepam	33.55	3818
Oxazepam	38.65	3242
Lorazepam	35.47[c]	68 284

[a] The concentration of each compound was $1.0\cdot10^{-4}$ mol l^{-1}. 0.05 mol l^{-1} borax with 0.002 mol l^{-1} CTAB, pH adjusted to 2.2 with orthophosphoric acid was used as buffer.
[b] In a mix with diazepam.
[c] In a mix with chlordiazepoxide.

in the sample plug results in a higher electric field existing in the sample plug compared with the previous sample. This therefore causes the cations to move much faster when the potential is applied and they effectively form a much tighter stack at the more distinct boundary between sample plug and run buffer in the capillary thus exhibiting a sharper and more efficient peak.

3.4.1. Calibration data

Using the above studies as a precursor some calibration data and RSD values were generated for the determination of diazepam by LVSS-CE. Two× 30 s hydrodynamic injections, a stacking potential of +12.5 kV and a separation potential of −12.5 kV were used throughout.

A calibration graph was constructed in the range $1.0\cdot10^{-4}$ mol l^{-1} to $2.5\cdot10^{-7}$ mol l^{-1} diazepam ($n=6$) and a correlation coefficient (R^2) of 0.9997 achieved. A typical electropherogram for a $5.0\cdot10^{-6}$ mol l^{-1} sample of diazepam by LVSS-CE is shown in Fig. 2.

In order to determine the RSD values for this technique a solution of $1.0\cdot10^{-6}$ mol l^{-1} diazepam was prepared and 2×30 s hydrodynamic injections made. Six replicate injections were then made and resulted in RSD for peak area and migration time of 3.17% and 4.09%, respectively.

The LOD was taken as three-times the blank signal at the migration time where diazepam normally occurs. This corresponded to a concentration of $5.8\cdot10^{-8}$ mol l^{-1}, approximately two-orders of magnitude lower than in conventional CE.

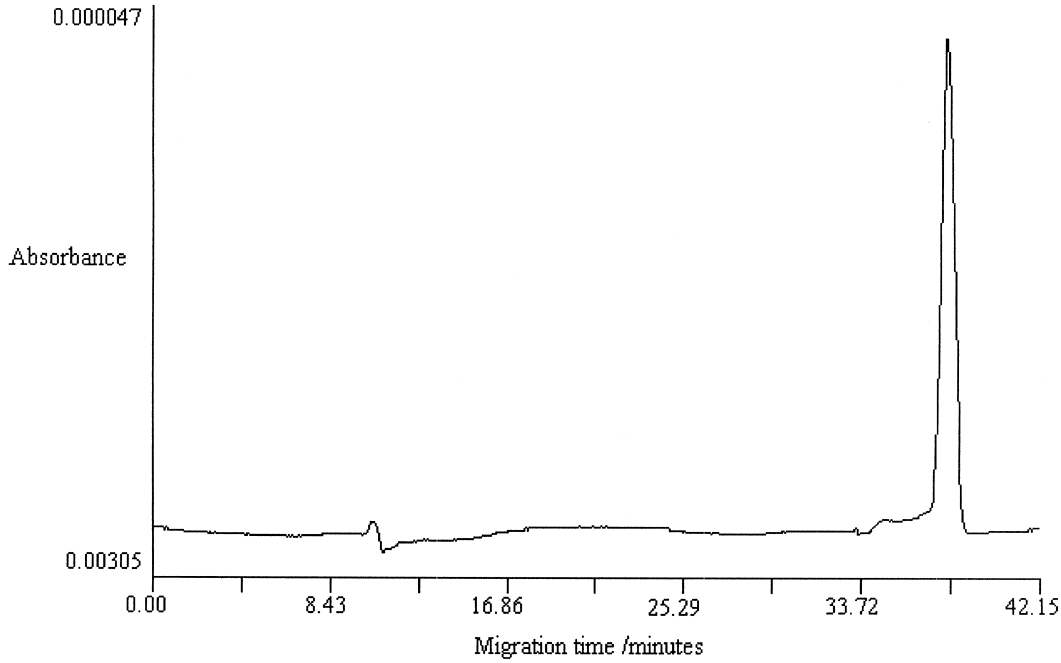


Fig. 2. LVSS-CE electropherogram of a $5.0\cdot10^{-6}$ mol l^{-1} diazepam sample prepared in 1% run buffer. Two×30 s hydrodynamic injections were made and a separation potential of −12.5 kV used after a stacking potential of 12.5 kV had been applied. The run buffer contained 0.05 mol l^{-1} sodium tetraborate and 0.002 mol l^{-1} CTAB adjusted to pH 2.2 with orthophosphoric acid.

For the determination of 1,4-benzodiazepines in mixtures spurious results were produced. It was observed that complex stacking phenomena contributed to what could only be described as preferential stacking of the analytes in mixtures. This was particularly evident in equimolar mixtures containing diazepam that consistently had a superior signal to the other compounds present. While the LOD is significantly lower in LVSS-CE the phenomena of shifting migration time hampers meaningful UV detection. This problem would be overcome if MS detection were used but a further problem is faced in that involatile salts and complexing agents such as phosphate and CTAB (vital for LVSS to occur) are incompatible with the electrospray interface and would cause contamination of the source. LVSS-CE–UV fast scanning detection may be one possible solution to the determination of these compounds in a matrix such as human hair by comparing the spectra of observed compounds to those in a previously constructed library. However, a more satisfactory solution may be found in the recent advent of orthogonal sampling devices for attachment to electrospray sources. These divert all unwanted salts and complexing agents to waste while the ions of interest are sampled into the source at right angles. This would therefore permit the use of LVSS-CE–MS of the benzodiazepines and allow the necessary LODs to be achieved.

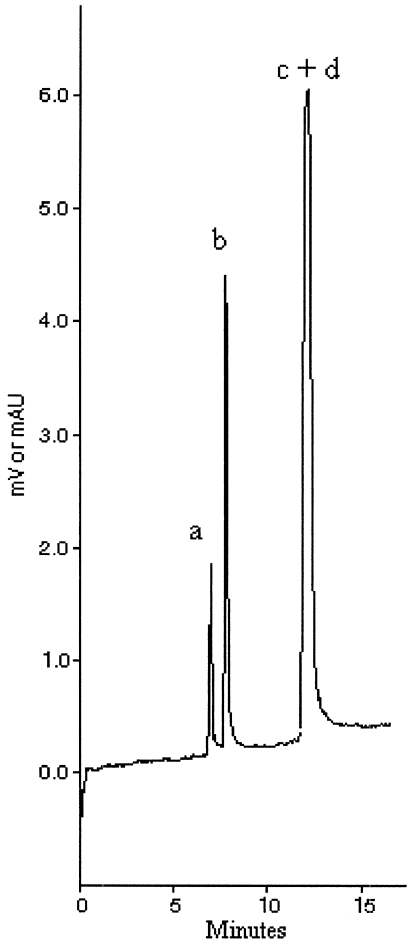


Fig. 3. (a) CE migration pattern of *N′*-desmethyldiazepam (a), diazepam (b), oxazepam and temazepam (c+d) as seen by the in-line UV–Vis detector during CE–MS determination of the four compounds. Injection point to UV detector=34 cm. (b) CE–ESI-MS migration pattern of diazepam and its metabolites. Injection point to MS detector=95 cm. The MS detector was set to detect the $[M+H]^+$ ions i.e., 273.2, 285.2, 287.2 and 301.0 for a–d, respectively. The oxazepam scan exhibits a two-peak profile possibly due to the nearness of its $[M+H]^+$ signal to that of diazepam. The second peak is therefore used as the analytical signal for oxazepam. Run buffer=0.02 mol l^{-1} citric acid and 15% methanol. A $2.5 \cdot 10^{-3}$ mol l^{-1} sample of the compounds was injected for 1.25 s at $1.103 \cdot 10^{-2}$ MPa.

3.5. CE–MS

Under the conditions used in these studies *N′*-desmethyldiazepam and diazepam migrate through the capillary to the UV detector in 6.89 and 7.72 min, respectively. Oxazepam and temazepam, which previously exhibited fused peaks in conventional CE, now co-migrate at around 11.97 min (Fig. 3a). The time taken to migrate to the MS detector is 19.19 and 21.31 min for *N′*-desmethyldiazepam and diazepam, and 32.75 and 33.36 min for oxazepam and temazepam, respectively (Fig. 3b). In this case the detector was set in MS mode to detect the $[M+H]^+$ ions, i.e., 273.2, 285.2, 287.2, 301.0 for *N′*-desmethyldiazepam, diazepam, oxazepam and temazepam, respectively. A two-peak profile is observed for oxazepam when determined in the presence of diazepam. The first observed peak is possibly due to a duplication of the diazepam signal because of the similarity in $[M+H]^+$ values for the two compounds. Once again however variance in migration time was not uncommon and is probably exaggerated due to

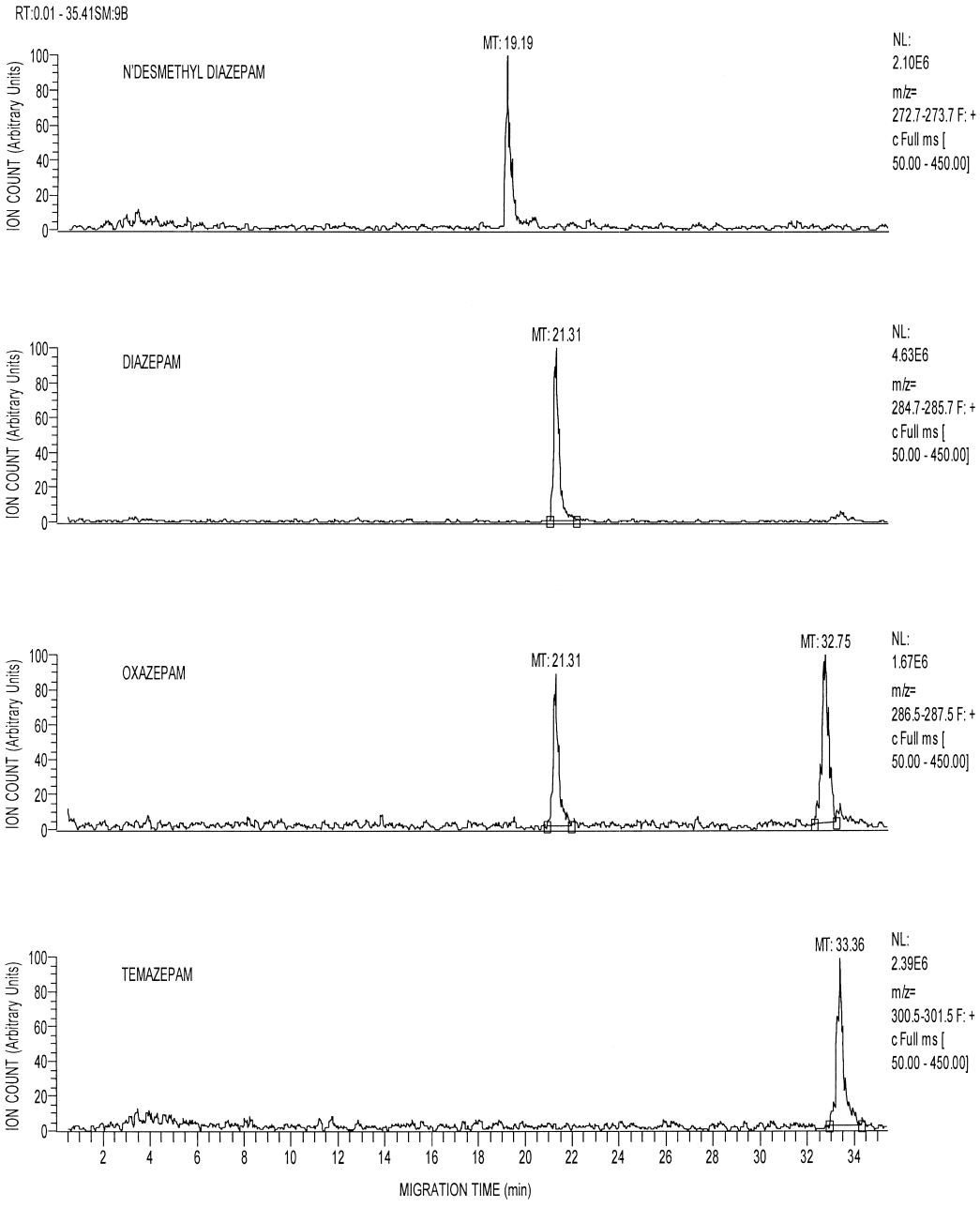


Fig. 3. (*continued*)

the complexities of harnessing two independent power supplies to give the required electrophoretic potential and the problems encountered in maintaining a stable spray current in the ESI source.

LODs for this technique were established by serially diluting standards until a predetermined S/N ratio, specified by the integration software was reached. For N'-desmethyldiazepam $S/N=3$ while for the other compounds the ratio was set to 2. MS–MS mode was used for these determinations.

This resulted in LODs of $1.0 \cdot 10^{-5}$ mol l^{-1} for oxazepam and temazepam, $9.0 \cdot 10^{-6}$ mol l^{-3} for diazepam and $8.0 \cdot 10^{-6}$ mol l^{-1} for *N′*-desmethyldiazepam.

The complexities of maintaining a stable spray current has a detrimental effect on the peak area recorded, as varying current seems to correlate with varying ionisation efficiency of the compounds. This is therefore manifested in very poor correlation coefficients for calibration data, even when the peak area measurements are made relative to an internal standard (7-aminonitrazepam). In the range $2.5 \cdot 10^{-4}$ to $2.5 \cdot 10^{-3}$ mol l^{-1} these were 0.8809, 0.6130, 0.8718 and 0.9674 for *N′*-desmethyldiazepam, diazepam, oxazepam and temazepam, respectively. The RSD values for a $1.0 \cdot 10^{-3}$ mol l^{-1} mixture of the four compounds were 4.44, 4.94, 6.35 and 6.43% calculated on absolute migration time and 65.77, 34.21, 51.77 and 60.02% calculated on peak area for *N′*-desmethyldiazepam, diazepam, oxazepam and temazepam, respectively. Using an internal standard improved the RSD on migration time to 0.33, 0.48, 2.58 and 2.44% for the four drugs as listed above. It is hoped that the use of the internal standard method will also cause significant improvement to the RSD on peak area.

3.6. LC–MS investigations

LC–MS of the 15 1,4-benzodiazepines is shown in Fig. 1b, showing poorer efficiency of separation compared to those achieved by CE.

MS–MS characterisation of the 1,4-benzodiazepines provided the following data shown in Table 5, and a typical MS–MS scan of diazepam and its metabolites is provided in Fig. 4.

3.6.1. Family group analysis

On taking each family group of compounds in turn the LOD and RSD values were achieved using LC–MS–MS determinations as shown in Table 6. It should be noted that under the conditions given, the nitrazepam family of compounds produced peaks of very poor shape and this is manifested in low efficiency values. RSD values were calculated at the $5.0 \cdot 10^{-7}$ mol l^{-1} level. Calibration data for each of the compounds is provided in Table 7.

Peak efficiency values (plates) were calculated for all the compounds. LC peaks were calculated at the $5.0 \cdot 10^{-7}$ mol l^{-1} level while the CE peaks were determined at the $7.5 \cdot 10^{-5}$ mol l^{-1} level. A comparison of these values is shown in Table 8. The values quoted were calculated using the half-width

Table 5
ESI-MS and ESI-MS–MS data for 15 1,4-benzodiazepines[a]

Compound	MS peak (m/z)	MS–MS peak (m/z)
Diazepam	285.2	257.1
N-Desmethyldiazepam	273.2	216.0
Oxazepam	287.2	268.9
Temazepam	301.0	282.9
Chlordiazepoxide	300.0	283.0
Demoxepam	287.2	270.1, 180.1
Nitrazepam	282.2	236.2
7-Aminonitrazepam	252.2	224.2, 121.2
7-Acetamidonitrazepam	294.2	207.3
Flunitrazepam	314.2	268.2
3-Hydroxyflunitrazepam	300.2	254.2
Lorazepam	321.1	302.8
Flurazepam	388.1	315.2
N′-(2-Hydroxyethyl)flurazepam	333.1	315.1
N′ Desalkylflurazepam	289.1	261.1

[a] The MS–MS ions were generated due to fragmentation of the $[M+H]^+$ ion by collisions with He gas in the ion-trap. Twenty-five % of the available collision energy was used for all compounds apart from oxazepam, nordiazepam and temazepam for which it was lowered to 20%.

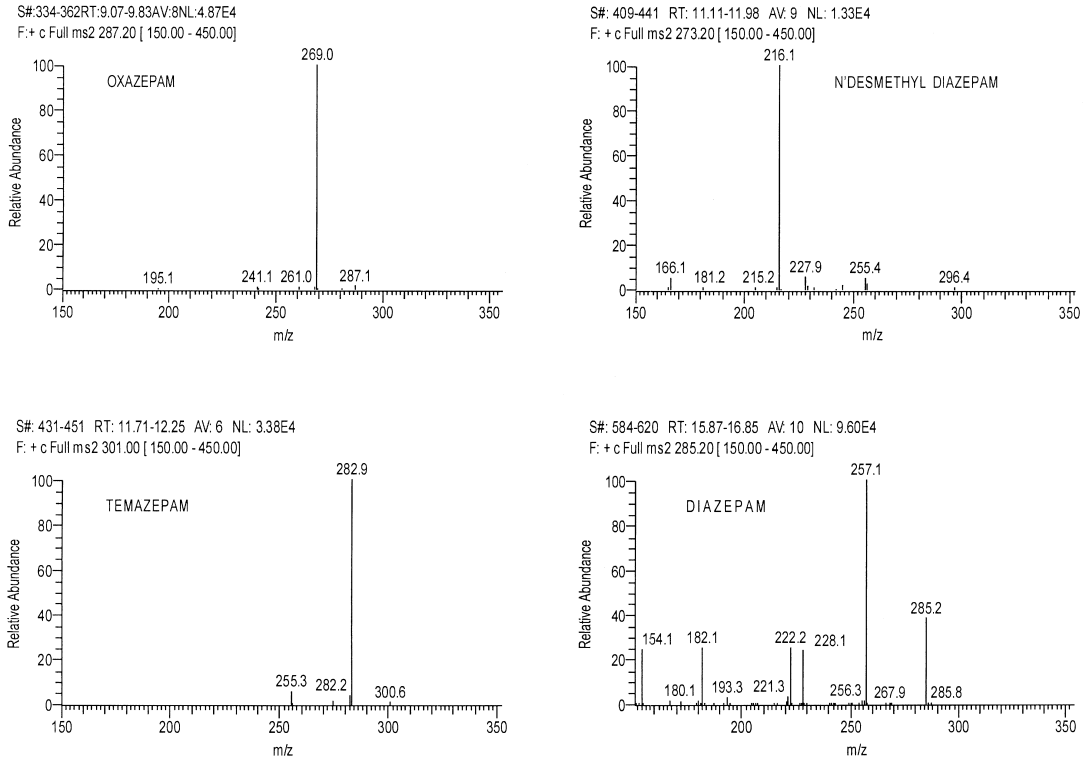


Fig. 4. Typical MS–MS spectra for *N′*-desmethyldiazepam, diazepam, oxazepam and temazepam; the parent ions fragmented to give these signals being 273.2, 285.2, 287.2 and 301.0, respectively.

method. It can be clearly seen and as one would expect, that CE offers much higher separation efficiency than LC in all cases.

A comparison of LOD values for CE–UV, CE–MS, LVSS-CE–UV and LC–MS–MS is provided in Table 9. Taking diazepam as an example, it can be

Table 6
LOD and RSD values for the 1,4-benzodiazepines studied by LC–MS–MS[a]

Compound	LOD ($\cdot 10^{-8}$ mol l^{-1})	Retention time (min)	RSD of retention time (%)	RSD of peak area or calculated concentration (%)
Diazepam	4.09	16.41	0.00	5.50
Oxazepam	4.40	9.58	0.57	4.45
Temazepam	5.57	12.36	0.00	6.11
N′-Desmethyldiazepam	9.13	11.51	0.47	4.99
Chlordiazepoxide	14.00	5.27	0.49	6.57
Demoxepam	15.48	4.12	1.99	9.58
Nitrazepam	1.30	9.93	0.35	8.55
7-Acetamidonitrazepam	2.21	7.20	1.11	30.21
7-Aminonitrazepam	3.41	6.90	3.33	42.80
Flurazepam	1.90	2.47	15.54	0.00
N′-(2-Hydroxyethyl)flurazepam	1.35	6.02	0.77	7.91
N′-Desalkylflurazepam	3.73	5.62	9.01	8.74
Flunitrazepam	2.67	6.13	0.43	22.98
3-Hydroxyflunitrazepam	5.91	4.91	0.08	21.96
Lorazepam	4.57	5.88	0.76	5.10

[a] The RSD values were determined at the $5.0 \cdot 10^{-7}$ mol l^{-1} level for each compound.

Table 7
Calibration data for the 1,4-benzodiazepines studied by LC–MS–MS

Compound	Correlation coefficient (R^2)	Range (mol l^{-1})	n
Diazepam	0.9983	$7.5\cdot10^{-8}$ to $5.0\cdot10^{-7}$	5
Oxazepam	0.9990	$7.5\cdot10^{-8}$ to $5.0\cdot10^{-7}$	5
Temazepam	0.9981	$7.5\cdot10^{-8}$ to $5.0\cdot10^{-7}$	5
N′-Desmethyldiazepam	0.9980	$1.0\cdot10^{-7}$ to $5.0\cdot10^{-7}$	4
Chlordiazepoxide	0.9985	$2.5\cdot10^{-7}$ to $2.5\cdot10^{-6}$	5
Demoxepam	0.9973	$2.5\cdot10^{-7}$ to $2.5\cdot10^{-6}$	5
Nitrazepam	1.0000	$1.0\cdot10^{-7}$ to $7.5\cdot10^{-7}$	3
7-Aectamidonitrazepam	0.9984	$2.5\cdot10^{-7}$ to $1.0\cdot10^{-6}$	4
7-Aminonitrazepam	0.9911	$7.5\cdot10^{-8}$ to $1.0\cdot10^{-6}$	3
Flurazepam	0.9944	$7.5\cdot10^{-8}$ to $1.0\cdot10^{-6}$	6
N′-(2-Hydroxyethyl)flurazepam	0.9930	$7.5\cdot10^{-8}$ to $1.0\cdot10^{-6}$	5
N′-Desalkylflurazepam	0.9961	$1.0\cdot10^{-7}$ to $1.0\cdot10^{-6}$	5
Flunitrazepam	0.9997	$1.0\cdot10^{-7}$ to $2.5\cdot10^{-6}$	5
3-Hydroxyflunitrazepam	0.9842	$1.0\cdot10^{-7}$ to $2.5\cdot10^{-6}$	6
Lorazepam	0.9959	$7.5\cdot10^{-8}$ to $1.0\cdot10^{-6}$	6

seen that LC–MS–MS has the lowest LOD closely followed by LVSS-CE–UV. Conventional CE with UV and MS detection both have high LODs in the 10^{-5}–10^{-6} mol l^{-1} range.

3.7. Hair analysis

Following Soxhlet extraction of the washed positive hair sample LC–MS–MS analysis was carried out and revealed levels of *N′*-desmethyldiazepam, diazepam, oxazepam and temazepam (following blank subtraction) as shown in Table 10. Diazepam, temazepam and oxazepam could be readily detected while *N′*-desmethyldiazepam, a metabolite of diazepam and many of the benzodiazepines, was just detectable in most cases, its found concentration being close to the LOD. The relatively low amount of analyte present in each case accounts for the relatively high RSD values. A typical ion-count chromatogram is provided in Fig. 5. A fuller account of this application will be provided in a future publication [46].

Table 8
Comparison of CE–UV and LC–MS–MS peak efficiencies[a]

Compound	LC efficiency	CE efficiency
Diazepam	10 101	72 857
Oxazepam	838	93 383
Temazepam	5621	98 536
N′-Desmethyldiazepam	4849	77 516
Chlordiazepoxide	9121	82 726
Demoxepam	349	27 221
Nitrazepam	18 763	70 800
7-Aectamidonitrazepam	174	82 562
7-Aminonitrazepam	204	84 867
Flurazepam	16 706	78 509
N′-(2-Hydroxyethyl)flurazepam	4399	50 634
N′-Desalkylflurazepam	16 756	60 502
Flunitrazepam	6798	22 393
3-Hydroxyflunitrazepam	4372	23 489
Lorazepam	10 833	21 332

[a] The CE compounds were determined at the $7.5 \cdot 10^{-5}$ mol l^{-1} level while the LC analysis took place at the $5.0 \cdot 10^{-7}$ mol l^{-1} level.

Table 9
Comparison of LODs for the diazepam family of compounds by CE–UV, CE–MS, LVSS-CE–UV and LC–MS–MS[a]

Compound	LOD (mol l^{-1})			
	CE–UV	CE–MS	LVSS-CE–UV	LC–MS–MS
Diazepam	$5.4 \cdot 10^{-6}$	$9.0 \cdot 10^{-6}$	$5.8 \cdot 10^{-8}$	$4.1 \cdot 10^{-8}$
Oxazepam	$1.3 \cdot 10^{-6}$	$1.0 \cdot 10^{-5}$	–	$4.4 \cdot 10^{-8}$
Temazepam	$2.0 \cdot 10^{-6}$	$1.0 \cdot 10^{-5}$	–	$5.6 \cdot 10^{-8}$
N′-Desmethyldiazepam	$1.6 \cdot 10^{-6}$	$8.0 \cdot 10^{-6}$	–	$9.1 \cdot 10^{-8}$

[a] LOD defined as the concentration resulting in a signal magnitude as follows; CE–UV, 3×SD blank signal; CE–MS–MS, $S/N=2$ or 3; LVSS-CE–UV, 3×blank signal; LC–MS–MS, 2×blank signal.

Table 10
Levels of drugs found in the Soxhlet extracted hair sample[a]

Compound	Measured concentration (μmol l^{-1})	Concentration (ng per mg hair)	RSD for measurement (%) ($n=6$)
Diazepam	0.492	1.23	16.50
Temazepam	0.363	0.96	24.75
Oxazepam	0.183	0.47	22.48
N′-Desmethyldiazepam	LOD	–	10.35

[a] The mobile phase was acetonitrile–water (65:35) and a flow-rate of 0.3 ml min^{-1} was used. A 20-μl injection was made.

4. Conclusions

It may be concluded therefore that while CE remains superior to LC in terms of separation efficiency, LC–MS has proved the most useful tool in sensitivity terms for identification of ng mg^{-1} levels of 1,4-benzodiazepines in human hair. MS–MS characterisation of the compounds under investigation enhances the selectivity of the technique thereby providing strong identification for each drug. The LODs produced in LC–MS–MS are acceptable for routine analysis in both clinical and forensic applications. For the determination of these compounds by CE–MS further study must be carried out to significantly improve signal reproducibility and LOD before the technique can be considered for

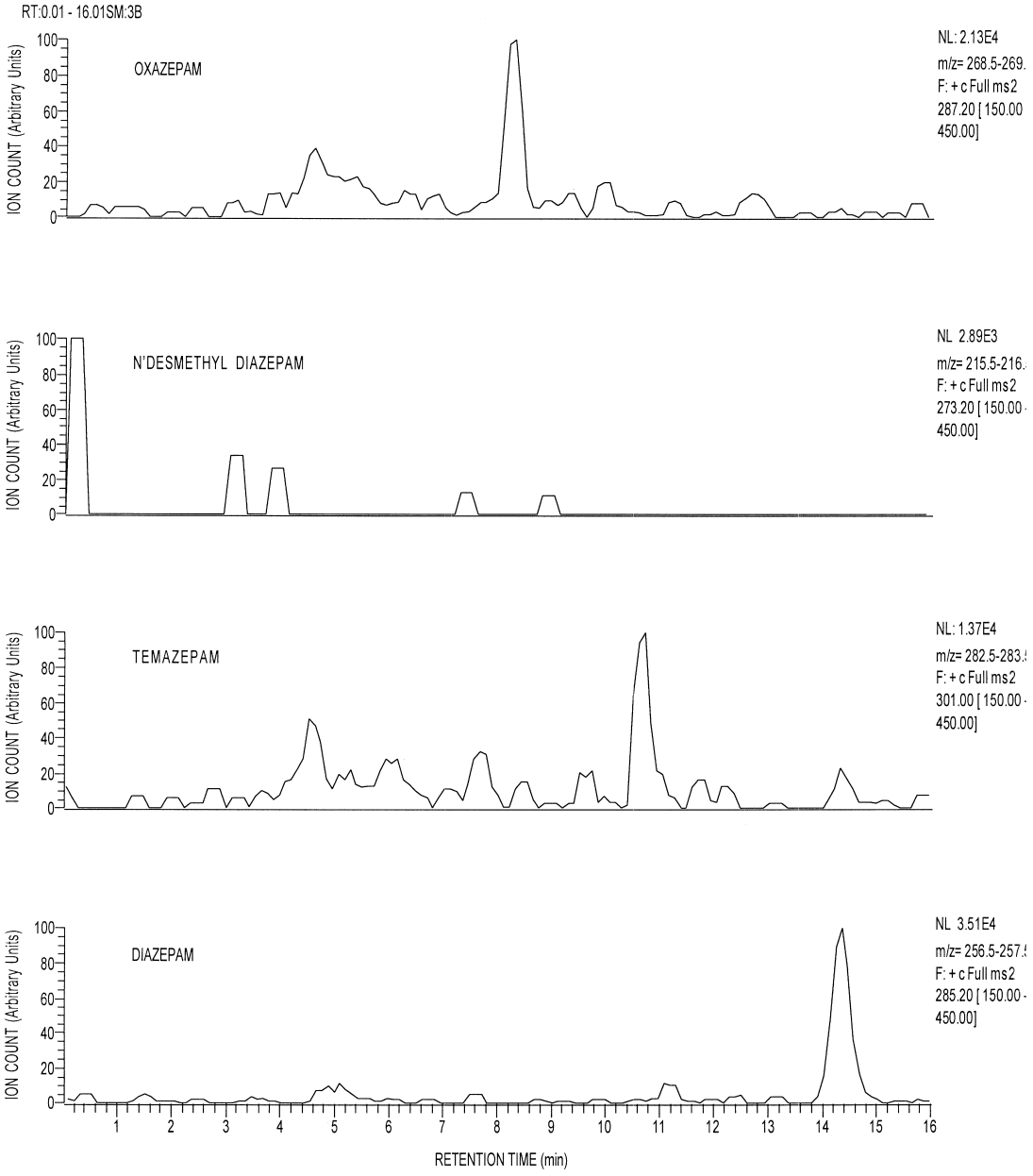


Fig. 5. LC–MS–MS of a positive hair sample from a patient treated with diazepam and temazepam. Mobile phase acetonitrile–water (65:35), Luna C_{18} 150 mm column, 20 μl injection, flow-rate 0.3 ml min^{-1}.

routine trace analysis. LVSS-CE–UV offers a significant lowering of the LOD compared with conventional CE, but the problem of shifting migration time hampers the use of this technique for routine analysis in complex matrices. This may be overcome by using fast-scanning UV detection or by incorporating orthogonal sampling apparatus onto the ESI source so that MS detection can take place.

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