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Review

Recent studies on the electrospray ionisation mass spectrometric behaviour of selected nitrogen-containing drug molecules and its application to drug analysis using liquid chromatography–electrospray ionisation mass spectrometry

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Abstract

This review presents recent studies on the electrospray ionisation mass spectrometry (ESI-MS) of selected N-containing drug molecules, their metabolites, formulation degradation products and process impurities taken from both studies in the author's laboratory and the recent literature using the Web of Knowledge database. Molecules of mass less than 500 Da are chosen according to selected structural classes in which they give ESI signals primarily in the positive ion mode as $[M + H]^+$ ions. The structural classes are drugs with amine-containing side chains, drugs with N-containing saturated ring structures, drugs with N-containing unsaturated ring structures and quaternary ammonium drugs. Details are given on the fragmentations, where available, that these ionic species exhibit in-source and in ion-trap, triple quadrupole and time-of flight mass spectrometers. Fragmentation data, again where available, using electron impact mass spectrometry (EI-MS) is included for comparison. A review of applications for the period 2004–2005, again taken from the Web of Knowledge database, of the technique liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS) to the detection and determination of these N-containing drug molecules in biomatrices, pharmaceutical formulations, etc., is then made. Analytical information on, for example, sample concentration techniques, LC separation conditions, recoveries from biological media, degradation products and limits of detection (LODs) are provided. Comparisons, where available, are also made with rival analytical techniques such as gas liquid chromatography–mass spectrometry (GLC–MS), capillary electrophoresis–electrospray ionisation mass spectrometry (CE–ESI-MS) and stripping voltammetry (SV).

Keywords: Electrospray ionisation mass spectrometry (ESI-MS); Fragmentation patterns; Drug analysis by liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS); Drugs of small molecular mass; Drug analysis by gas liquid chromatography–mass spectrometry (GLC–MS); Drug analysis by capillary electrophoresis–electrospray ionisation mass spectrometry (CE–ESI-MS); Stripping voltammetry (SV)

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1. Introduction

Drugs, their metabolites, formulation degradation products and process impurities have been subjected to increasing investigation by electrospray ionisation mass spectrometry (ESI-MS) since the technique was introduced by Yamashita and Fenn [1] in 1984 with a significant commercial impact having been made since 1990. This invention was recognised by The Royal Swedish Academy of Sciences with the award of The Nobel Prize in Chemistry for 2002 partly to John B. Fenn for his pioneering work in ESI-MS. The Web of Knowledge database reveals an increasing use of ESI-MS for drug analysis with 53, 64, 76, 101 publications in the years 2001–2004, respectively. Detailed structural information on drugs and their related molecules can be obtained by resort to cone voltage fragmentation with a single MS instrument, collisionally induced dissociation (CID) with triple quadrupole MS instruments, MS^n techniques using quadrupole ion-trap instrumentation and time-of-flight mass spectrometry (ToFMS). ESI has now become the most important ionisation technique for the on-line coupling of liquid phase separation techniques such as LC with MS. The above mentioned database also reveals 29, 35, 55, 72 publications in the years 2001–2004, respectively, on the liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS) of drug molecules. Perusal of these database publications shows how LC–ESI-MS is being used for the analysis of a wide variety of drug molecules in matrices such as body fluids, foods, natural waters, pharmaceutical formulations, etc.

There have been several recent reviews on the application of LC–MS in general and LC–ESI-MS in particular to the identification and determination of small molecules of clinical, forensic and pharmaceutical significance. Marquet [2] has reported on the progress of LC–MS in clinical and forensic toxicology and has stated that there is an increased use of this technique using atmospheric pressure ionisation (API) in pharmacokinetics, toxicology and therapeutic drug monitoring (TDM). It is now currently competing with gas liquid chromatography–mass spectrometry (GLC–MS) for the status of the reference analytical technique in toxicology. The advent of robust and analytically reliable combinations of LC separation with MS detection that possess a high throughput of samples has opened new perspectives in terms of MS identification of small molecules (e.g., polar metabolites) or biopolymers of toxicological relevance that are difficult to analyse by GLC–MS. This review underlined the large number and variety of drugs or classes of drugs (e.g., drugs of abuse, therapeutic drugs) or toxic compounds (e.g., pesticides) that can be readily determined using such instruments, the respective merits of the different ionisation sources such as ESI and the improvements brought about by tandem MS (MS/MS). This review also discussed new applications of LC–MS in the field of toxicology, such as “general unknown” screening procedures and mass spectral libraries using LC–API-MS or MS/MS, presenting the different solutions proposed to overcome the naturally low fragmentation using API sources. Finally, the opportunities afforded by the most recent or proposed instrument designs were addressed. A further publication from Marquet and coworkers [3] dealt with an evaluation of this “general unknown” screening procedure using LC–ESI-MS and compared it to LC-diode array detection (DAD) and GLC. Using solid-phase extraction (SPE), the LC–MS method identified 75% of unknown compounds contained in 51 serum samples (versus 66% for GLC–MS and 71% for LC-DAD), including 8% that the other two techniques failed to identify (versus 8% for GLC–MS and 9.5% for LC-DAD). Therefore, the technique was stated to be complementary to GLC–MS and/or LC-DAD and helped enlarge the range of drugs detected in clinical toxicology. LC–ESI-MS could also be useful in forensic toxicology to confirm a positive result as 38% of all the compounds were detected by the three techniques and 36% by two of them.

The applications of LC–API-MS in routine forensic toxicological casework have been presented by Bogusz [4]. This technique has been used for routine determination of several groups of drugs such as opiate agonists (e.g., morphine, codeine, dihydrocodeine and their glucuronides, methadone,

buprenorphine), cocaine and its metabolites (benzoylecgonine and ecgonine methyl ester), amphetamine and other psychoactive phenethylamines (e.g., MDMA, MDE or MDA), benzodiazepine derivatives (flunitrazepam and metabolites, triazolam, bromazepam), hallucinogens (LSD, psilocybin, psilocin) and olanzapine (OLZ). A common solid-phase extraction (SPE) procedure for all drugs (with the exception of LSD) was also developed. ESI was found to be particularly sensitive to polar drugs such as psilocin or psilocybin. Bogusz [4] stated that the use of these techniques for general screening depended on the establishment of interlaboratory databases of standardised mass spectra.

Maurer has reviewed procedures for quantification of drugs in blood, plasma, serum, or oral fluid (saliva, etc.) using LC–MS and LC–MS/MS [5] these procedures being important prerequisites for competent toxicological judgments in clinical and forensic toxicology. They cover blood (plasma, serum) analysis of amphetamines and related designer drugs, anesthetics, anticonvulsants, benzodiazepines, opioids, serotonergic drugs, tricyclic antidepressants, neuroleptics, antihistamines, beta-blockers, muscle relaxants, sulphonylurea-type antidiabetics and oral fluid analysis of amphetamines and related designer drugs, cocaine, benzoylecgonine, codeine, morphine, enantiomers of methadone and its main metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), the nicotine metabolites cotinine and hydroxycotinine and finally risperidone (RIP) and its metabolite 9-hydroxyrisperidone. The LC–MS procedures are critically discussed and this includes sample work-up and ion suppression effects.

Heftmann has recently edited “Applications of Liquid Chromatography coupled to Mass Spectrometry in Pharmacology” [6] which contains some relevant reviews on LC–ESI-MS and its applications to the detection and determination of small molecules of clinical, forensic and pharmaceutical significance. For example, in this volume, Smyth [7] has reviewed the ESI-MS behaviour of selected drugs, essentially from the publication period 1993 up to 2000. In particular, details were given on the fragmentations that the ionic species exhibited. A table giving mass losses/signals at low *m*/*z* values was presented in this review which would be of value in the characterisation of unknown metabolites and also natural product pharmaceuticals isolated from plants, for example. This has been updated recently and given in this review as Table 1.

Watson et al. [8] have included the analysis of antioxidant xenobiotics in biological systems in their review of the applications of MS with sample introduction in the liquid phase to the analysis of the oxidation products of DNA, proteins and lipids in vitro and in vivo. The most commonly used MS mode in this area of study was reported to be ESI-MS that could also include CID.

Rivier [9], also in this Heftmann volume [6], has stated that, in contrast to GLC–MS, many factors influence MS of organic compounds when analyzed by LC–MS. In-source fragmentation or CID using electrospray ionisation or atmospheric pressure chemical ionisation have demonstrated the need for the standardisation of experimental conditions when assembling a library of reference spectra. Rivier [9] has further indicated that magnetic sector, quadrupole, ion trap or time-of-flight filters placed on single or multiple mass spectrometry instruments coupled with LC deliver unique mass intensity patterns from the same molecule that are most often not readily comparable between each other. This has therefore made it very difficult or even impossible to rely on any collections of reference data whatever comparison algorithm is applied for library searching or using manual comparison when full confirmation of identity is required for a court’s scrutiny, for example. However, it is still possible in these cases to obtain sufficient MS information about the unknown molecule and the reference substance providing a particular instrument/technique is employed with standardised experimental conditions. In this review, this problem was discussed with reference to forensic toxicology and doping control in sports. Several official guidelines have been released recently and await their application in actual cases. Closely related areas like residues in living animals and meat products are introducing regulations with detailed procedures. Basically, they propose the accumulation of a finite quantity of information about the unknown molecule and the reference substance. The article concluded with an evaluation of these confirmatory guidelines for their application in LC–MS.

Following on from Rivier’s publication, Gergov et al. [10] have recently studied the reproducibility of product ion spectra acquired using a liquid chromatography triple-quadrupole tandem mass spectrometry instrument over a 4-year period and also using three other LC–MS/MS instruments, one from the same manufacturer and two from a different manufacturer. The MS/MS spectra of 30 drug substances were generated in positive ESI mode at low, medium, and high collision energies (20, 35 and 50 eV). Purity and Fit score percentages against a 400-compound LC–MS/MS library were calculated using an algorithm in which fragment intensity ratios and weighting factors were included. The long-term reproducibility study was conducted using a brand A instrument which showed that after 4 years the reproducibility of the product ion spectra was still 94%, expressed as average Purity score. The inter-laboratory study involved two aspects. Firstly, two LC–MS/MS spectral libraries, created independently in separate laboratories using brand A instruments, were compared with each other. The average Fit and Purity scores of spectra from one library against the other were better than 93 and 91%, respectively, when the same collision energies were used. Secondly, for the comparison of product ion spectra between brand A and brand B instruments, fragmentation conditions were first standardised for amitriptyline as the standard analyte. The average Fit scores of brand B spectra against the brand A spectral library varied between 79 and 85% at all three collision energies. The authors stated that their results indicated that, after standardising the instrumental conditions, LC–MS/MS spectral libraries of drug substances would be suitable for inter-laboratory use.

Table 1
Mass losses/signals at low *m*/*z* values using ESI-MS and structural inferences

Mass loss/signal at low *m*/*z* value	Structural inference	Examples
15	Loss of CH_3 from methoxy-substituted aromatics	Coumarins and quinolines, narcotine
	Loss of CH_3 substituent from aromatic ring	Zolpidem, olanzapine
17	Loss of NH_3 from end-of-chain NH_2 group with at least two adjacent CH_2 groups	Amphetamine, 5-HT
17	Loss of NH_3 from pyrrolidine ring	Nornicotine
17	Loss of NH_3 from aromatic NH_2 group	2,6-Xylidine (lignocaine metabolite)
17	Loss of OH from *N*-oxide	Chlordiazepoxide
18	Loss of H_2O from aliphatic OH	Clenbuterol, salbutamol, morphine, codeine, chloramphenicol, 3-OH-benzodiazepines, quinolines, yohimbine
20	Loss of HF	7-Aminoflunitrazepam, *N*-desmethylflunitrazepam
28	Loss of $CH_2{=}CH_2$ after loss of adjacent end-of-chain amine	Chlorpromazine
28	Loss of $CH_2{=}CH_2$ from ethyl ester substituent	Pethidine
28	Loss of $CH_2{=}CH_2$ from aromatic ethyl ether	Sildenafil
28	Loss of CO/NCH_2 with ring contraction	Benzodiazepines, zopiclone and *N*-desmethyl metabolite, coumarins, quinolines
29	Loss of $COH/CH_2{=}NH$ with ring contraction	Benzodiazepines, e.g., flunitrazepam
31	Loss of CH_3NH_2 from end of chain	*N*′-Methyl 5-HT
31	Loss of CH_3NH_2 from CH_3-substituted pyrrolidine ring	Nicotine
31	Loss of OCH_3	Coumarins
32	Loss of S from cyclic structure	Chlorpromazine
32	Loss of CH_3OH from methyl ester substituent	Cocaine, reserpine
35/	Loss of aromatic Cl/HCl	Clenbuterol, benzodiazepines
42	Loss of $CH_3CH{=}CH_2$ after loss of end-of chain amine	Methadone trimpramine
42	Loss of C_3H_6	Coumarins
42	Loss of $CH_3CH{=}CH_2$ from $-NH-CH(CH_3)_2$ end-of-chain group	Propranol
42	Loss of ketene, $CH_2{=}C{=}O$	Coumarins, 7-acetamidonitrazepam
43	Loss of $CH_2{=}N{-}CH_3$	LSD and derivatives
44	Loss of CO_2 from ring	Lactones, e.g., coumarins
	Loss of CO_2 from COOH substituent	Penicillins
	Loss of CO_2 in molecule	Zopiclone
45	Loss of $(CH_3)_2NH$ from end-of-chain	Chlorpromazine, zolpidem, bufotenine
46	Loss of NO_2	7-NO_2-1,4-benzodiazepines, metronidazole
46	Loss of C_2H_5OH from ethyl ester substituent	Pethidine
56	Loss of $(CH_3)_2C{=}CH_2$ from chain end	Clenbuterol, salbutamol, quinolines
57	Loss of $CH_3CH{=}NCH_3$	6-MAM, codeine, morphine
58	Signal of $CH_2{=}N^+H(C_2H_5)$ from amine-containing carbon chain	Lignocaine and monoethyl metabolite
58	Loss of CH_3CONH	7-Acetamido-nitrazepam
58	Signal of $CH_2{=}N^+(CH_3)_2$ from end-of-chain	Trimpramine
59	Loss of $N(CH_3)_3$ from end-of-chain	5-HTQ
59	Loss of CH_3CONH_2	7-Acetamidonitrazepam
60	Loss of CH_3COOH as substituent	Coumarins, quinolines, 6-MAM
60	Loss of $HCOOCH_3$ from $-COOCH_3$ substituent	Cocaine
64	Loss of SO_2	Dyes
68	Loss of C_5H_8 as side chain	Coumarins and quinolines
73	Loss of $NH(C_2H_5)_2$ from end-of-chain	Flurazepam, procaine
74	Loss of $HCOOC_2H_5$ from $-COOC_2H_5$ substituent	Pethidine
79	Loss of Br as aromatic substituent	Br-substituted quinolines
80	Loss of SO_3	Dyes, sulphate conjugates
85	Loss of piperidine	CPT-11
86	Signal of $CH_2{=}N^+(C_2H_5)_2$ from amine-containing carbon chain	Lignocaine

Table 1 (*Continued*)

Mass loss/signal at low *m/z* value	Structural inference	Examples
86	Signal of $CH_2{=}CH{-}CH_2NH^+(CH_3)_2$ from amine-containing carbon chain	Chlorpromazine
100	Signal of $CH_2{=}CH(CH_3)CH_2NH^+(CH_3)_2$	Trimpramine
100	Signal of $CH_2{=}CHNH^+(C_2H_5)_2$	Procaine
101	Loss of OHC-N$(C_2H_5)_2$	LSD
122	Loss of C_6H_5COOH	Cocaine
124	Loss of C_9H_{16} from side chain	Quinolines
127	Loss of I	I-substituted coumarins
176	Loss of glucuronide from drug conjugate	Nitrocatechol, morphine

Searchable libraries of MS/MS spectra, obtained using LC–MS/MS with data-dependent scan mode switching on both quadrupole ion trap and triple-quadrupole mass spectrometers in conjunction with electrospray ionisation, have also been recently presented [11]. The effects on library search scores of changing the parameters for producing CID on both instrument types were systematically evaluated. The authors have stated that these observations have served as a basis for determining a universal set of conditions for building MS/MS libraries. A group of 19 closely related steroids was used in the study and the ability to obtain library-searchable spectra at low concentrations was demonstrated for the analysis of a sample of progesterone spiked with hydroxyprogesterone impurities at levels of 0.1 and 0.01%.

Maralikova and Weinmann [12] have recently noted that guidelines for confirmatory analysis by GLC–MS and LC–MS/MS have now been published by several organizations (WADA, IOC, SOFT, GTFCh, EU). However, these guidelines have not yet been included in procedures for drug analysis with LC–MS/MS. The prerequisites for forensic confirmatory analysis by LC–MS/MS with respect to EU guidelines are chromatographic separation, a minimum number of two MS/MS transitions and predefined thresholds for the variability of the relative intensities of the MS/MS transitions in samples and reference standards. LC–MS/MS methods for determination of several classes of drugs of abuse including some basic drugs (opiates, stimulants), cannabinoids and some of their phase-I- and phase-II-metabolites (especially glucuronides) in urine and serum of drug abusers, crime offenders and victims were developed and validated following the above recommendations and were presented in this paper. At least two multiple reaction monitoring (MRM) transitions for each substance were monitored to provide sufficient identification of drugs, deuterated analogues of analytes were used as internal standards for quantitation where possible and LC separation was performed on reversed-phase columns with gradient elution. Validation data obtained and the application to real samples showed that the requested criteria for confirmatory analysis of drugs of abuse by EU guidelines could be fulfilled with a total number of four identification points by LC–MS/MS methods using a triple-quadrupole mass spectrometer. Furthermore, the methods were found sufficiently sensitive to meet current requirements for confirmatory analysis of drugs of abuse in driving under the influence of drugs (DUID) cases established by the Society of Toxicological and Forensic Chemistry (GTFCh).

Halket et al. [13] have reviewed chemical derivatisation and mass spectral libraries in metabolic profiling by GLC–MS and LC–MS/MS. These two major hyphenated techniques employed in metabolic profiling complement direct fingerprinting methods such as atmospheric pressure ionisation (API) quadrupole time-of-flight MS, API Fourier transform MS and NMR. In GLC–MS, the analytes are normally derivatised and the EI mass spectra obtained are reproducible and suitable for library matching, mass spectral collections being readily available. In LC–MS, however, derivatisation and library matching were stated to be at an early stage of development with reference to mini-reviews being provided. Such derivatisation can dramatically increase the sensitivity and specificity of LC–MS methods for less polar compounds and can provide additional structural information. The potential of derivatisation for metabolic profiling in LC–MS was demonstrated by the enhanced analysis of plant extracts, including the potential to measure volatile acids such as formic acid, difficult to achieve by GLC–MS. The important role of mass spectral library creation and usage in these techniques is discussed and illustrated by examples.

Kudo et al. [14], again in the Heftman volume, have commented that, although GLC–MS and LC-diode array detection are mainly used in Japan for comprehensive screening and confirmation of drugs and poisons, the application of LC–MS in the field of forensic toxicology in Japan is expected to markedly increase in the future. Balizs and Hewitt [15] have described the applications of LC–MS/MS in the analysis of veterinary drug residues. They have reported that since the development of commercial atmospheric pressure interfaces LC–MS/MS has become widely used as a complementary technique to GLC–MS in residue analysis because of its applicability to the determination of polar and/or non-volatile compounds without derivatisation. This review included both ESI and APCI methods. Atcheson et al. [16] have reviewed drug measurement by LC–MS with particular reference to pharmacokinetic and pharmacodynamic studies.

Kostiainen et al. [17] have stated that both ESI and APCI, used in conjunction with LC, have become important and widely used methods in the analysis of metabolites owing to their superior efficiency, sensitivity and specificity. In this paper [17] the feasibility of LC–API-MS techniques in

the identification, structural characterisation and quantitation of drug metabolites has been reviewed. Sample preparation, LC techniques, isotope labelling, suitability of different MS techniques (such as MS/MS and high-resolution MS in drug metabolite analysis) have been discussed together with automation of data acquisition and interpretation.

A critical comparison of the techniques LC–ESI-MS and capillary electrophoresis–electrospray ionisation mass spectrometry (CE–ESI-MS) to the detection and determination of small molecules of significance in clinical and forensic science, as revealed in the literature up to 2003, has recently been published [18].

Dethy et al. [19] have demonstrated direct bioanalysis of drugs in plasma without the use of LC using nanoelectrospray infusion from a silicon chip coupled with tandem mass spectrometry. This has been achieved using an automated liquid sampler integrated with an array of microfabricated electrospray nozzles allowing rapid, serial sample introduction (1 min/sample). Standard curves prepared in human plasma for verapamil ($R^2 = 0.999$) and its metabolite norverapamil ($R^2 = 0.998$) were linear over a range of 2.5–500 ng mL^{-1}. A limit of quantitation (LOQ) of 5 ng mL^{-1} was assigned for both analytes. Sample preparation consisted of protein precipitation with an organic solvent containing the structural analogue gallopamil as an internal standard. Protein precipitation was selected both to maximise throughput and to test the robustness of direct nanoelectrospray infusion. Aliquots of supernatant (10 μL) were transferred to the back plane of the chip using disposable, conductive pipet tips for direct infusion at a flow rate of 300 nL min^{-1}. ESI occurred from the etched nozzles (30 μm o.d.) on the front of the chip, initiated by a voltage applied to the liquid through the pipet tip. The chip was positioned near the API sampling orifice of a triple quadrupole mass spectrometer which was operated in single/selected reaction monitoring (SRM) mode. Results were presented that documented the complete elimination of system carry-over, attributed to lack of a redundant fluid path. This technology was stated to offer potential advantages for MS-based screening applications in drug discovery by reducing the time for methods development and sample analysis.

Sheen and Her [20] have experimented with the rarely used negative ion electrospray mode in drug identification/determination for the analysis of neutral drugs in human plasma by fluoride attachment in LC–ESI-MS/MS. The analyses of several neutral drugs, mephenesin, guaifenesin, simvastatin, podophyllotoxin and inositol, were carried out by negative ion mode ESI-MS using adduct formation with three different halide ions [20]. The fluoride, chloride and bromide adducts of the selected drugs exhibited intense signals in negative ion mode ESI-MS. Under collision-induced dissociation, the major product ions of bromide and chloride adducts were the nonspecific bromide and chloride anions, respectively. In contrast, fluoride adducts produced strong $[M - H]^-$ ions as well as intense product ions. This technique was applied to the analysis of these selected neutral drugs in human plasma. LODs in the range of 0.025–0.05 ng mL^{-1} were achieved using 0.5 mL plasma. Good linearity was observed for each of the drugs examined in human plasma over the range of 0.05–50 ng mL^{-1}.

p-Toluenesulfonyl isocyanate has been used as a novel derivatisation reagent to enhance electrospray ionisation in its application to the LC–MS determination of two stereoisomers of 3-hydroxyl-7-methyl-norethynodrel in plasma, again using the negative ion mode [21]. The derivatisation for the two pharmacologically active 3-hydroxyl metabolites, 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel by *p*-toluenesulfonyl isocyanate was accomplished in 2 min at room temperature. The off-line derivatisation procedure introduced an easily ionisable sulfonylcarbamic ester moiety to the metabolites. This greatly improved the analytes' sensitivity in the negative ion mode and enabled the authors to achieve the desired LOQ of 100 pg/mL in plasma. The method could be used for the pharmacokinetic study of tibolone in human subjects.

This review itself presents recent studies on the electrospray ionisation mass spectrometry (ESI-MS) of selected N-containing drug molecules, their metabolites, formulation degradation products and process impurities taken from both studies in the author's laboratory and the recent literature using the Web of Knowledge database. Molecules of mass less than 500 Da are chosen according to selected structural classes in which they give ESI signals primarily as $[M + H]^+$ ions. The structural classes are drugs with amine-containing side chains, drugs with N-containing saturated ring structures, drugs with N-containing unsaturated ring structures and quaternary ammonium drugs. Details are given on the fragmentations, where available, that these ionic species exhibit in-source and in ion-trap, triple quadrupole and time-of flight mass spectrometers. Fragmentation data, again where available, using electron impact mass spectrometry (EI-MS) is included for comparison.

A review of applications for the period 2004–2005, again taken from the Web of Knowledge database, of the technique liquid chromatography–electrospray ionisation mass spectrometry to the detection and determination of these N-containing drug molecules in biomatrices, pharmaceutical formulations, etc., is then made. Analytical information on, for example, sample concentration techniques, LC separation conditions, recoveries from biological media, degradation products and limits of detection (LOD) are provided. Comparisons, where available, are also made with rival analytical techniques such as gas liquid chromatography–mass spectrometry, capillary electrophoresis–electrospray ionisation mass spectrometry and stripping voltammetry (SV).

2. Drugs with amine-containing side chains

Drugs with amine-containing side chains commonly protonate on the N-atom of the side chain in the ESI process. This can then be followed by fragmentation processes involving shortening of this side chain with the energetically favourable

Table 2
Heats of formation of neutral molecules formed in fragmentations following electrospray ionisation of drug molecules

Neutral molecule	ΔH_f° (kJ mol^{-1})
HF(g)	−271.1
HCl(g)	−92.3
H_2O(g)	−241.8
SO_2(g)	−296.8
NH_3(g)	−46.1
NO_2(g)	+33.2
CO(g)	−110.5
CO_2(g)	−393.5
C_5H_{12}(g)	−146.4
$CH_3COOC_2H_5$(l)	−486.6
CH_3NH_2(g)	−28.0
$C_2H_5NH_2$(g)	−48.5

elimination of neutral molecules such as amines, alkenes, H_2O, etc., (Table 2) and also contractions to ring systems to which these side chains are bonded. Such processes will be illustrated under the following sub headings.

2.1. Phenothiazines

The phenothiazine class of pharmaceutical compounds has long been used for the treatment of mental disorders such as schizophrenia. The phenothiazine, chlorpromazine, when studied by ESI-MS^n [22], lost 45 U as the neutral secondary amine $HN(CH_3)_2$ at MS^2 in a charge site initiated fragmentation of the relatively weak C–N bond (bond energy 293 kJ mol^{-1}) with hydrogen atom transfer to form a shortened chain ending in $–CH_2=CH_2$ with a signal at *m/z* 274.0 (Fig. 1). The loss of such amines is modestly energetically favourable with values of ΔH_f° quoted as −28.0 and −48.5 kJ mol^{-1} for methylamine(g) and ethylamine(g), respectively (Table 2). This alkene is subsequently lost as 28u at MS^3 yielding a signal at *m/z* 246.1. A sulphur atom is then lost from the ring system resulting in a signal at *m/z* 214.3 using MS^3 and MS^4. Furthermore, the C–N bond which involves the N atom of the tricyclic structure can also break by in-source fragmentation yielding $CH_2=CH–CH_2–NH^+(CH_3)_2$ at *m/z* 86.1 for chlorpromazine. The QToF-MS/MS behaviour of chlorpromazine has supported by elemental analysis these proposed fragmentation products [22]. In this paper [22], Joyce et al. have established rules of fragmentation for such drugs with a carbon chain ending in a tertiary nitrogen atom with at least two methylene or substituted methylene groups separating this nitrogen atom from the other end of the carbon chain. These drugs will lose the end nitrogen atom as the corresponding secondary amine in both in-source fragmentation (MS) and the MS^2 mode. The deaminated ions will then lose the corresponding alkene formed from these two methylene or substituted methylene groups using MS and MS^n modes. This is exemplified by the ESI-MS^n behaviour of chlorpromazine and other drugs such as trimpramine and methadone, discussed in Sections 2.5 and 2.6.

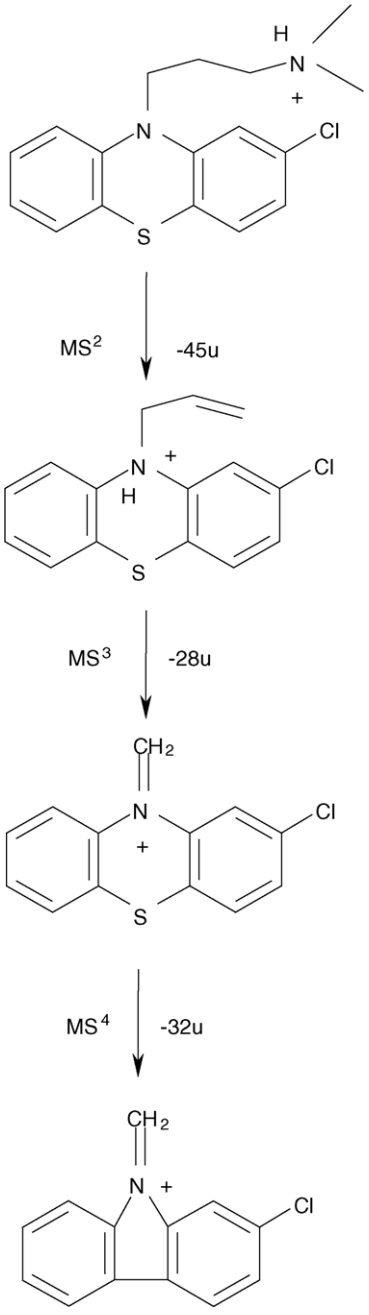

Fig. 1. MS^n fragmentation pattern for chlorpromazine.

Such ESI-MS^n and QToF-MS/MS behaviour should be compared to that observed by electron impact mass spectrometry (EI-MS) [23]. The phenothiazine, promethazine, with side chain $N–CH_2–CH(CH_3)–N(CH_3)_2$ fragments at the two N–C bonds to a minor degree but principally at the C–C bond yielding a base peak at *m/z* 72 due to $CH_3CH=N^+(CH_3)_2$. Promazine, with side-chain $–N–CH_2–CH_2–CH_2–N(CH_3)_2$, fragments at all of the shown bonds and yields major signals at *m/z* 86 and 58 (base peak), the former signal being observed for chlorpromazine using ESI-MS^n and QToF-MS/MS. These marker signals for amine-containing side chains at relatively low *m/z* values are also observed for

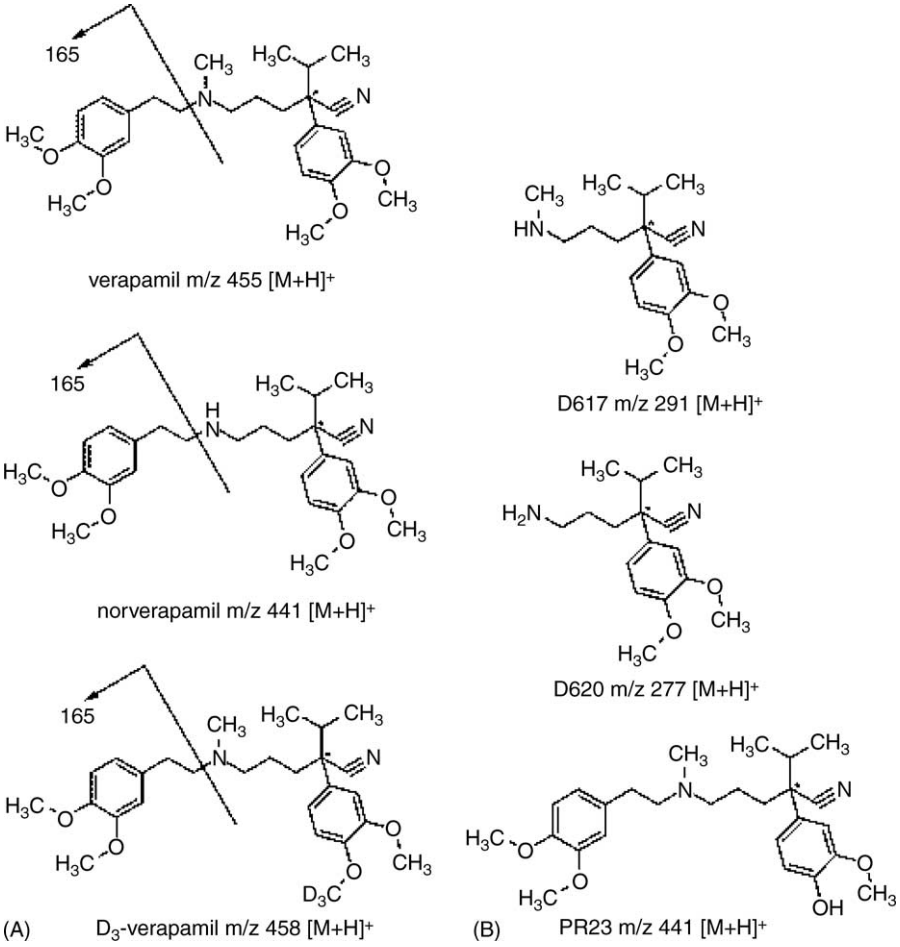

Fig. 2. (A) Structures of verapamil, norverapamil and D_3-verapamil (internal standard). The asymmetric carbons are marked with asterisks. The structures of the fragments corresponding to the daughter ions at *m/z* 165 are suggested. (B) Structures of the metabolites D617, D620 and PR23. The asymmetric carbons are marked with asterisks.

the phenothiazines, acepromazine and propionylpromazine, using EI-MS [23].

There have been no recent papers in 2004–2005 in the Web of Knowledge database concerning the application of LC–ESI-MS to the determination of phenothiazines.

2.2. Verapamil and its metabolites

Verapamil belongs to the pharmacological class of calcium channel blockers. It is used for the treatment of cardiovascular diseases like hypertension and angina pectoris. The substance has in its structure a tertiary amine with one asymmetric carbon atom and it can thus exist in two enantiomeric forms. However, verapamil is administered as a racemate despite documentation of stereogenic differences in pharmacological potency as well as in pharmacokinetics. It is extensively metabolised in the human body. The main metabolite with pharmacological activity is the *N*-demethylated form, i.e., norverapamil. The other main metabolites are D617, D620 and PR23. A stereoselective LC–ESI-MS/MS method for the simultaneous quantification of the enantiomers of verapamil and its active main metabolite norverapamil in human plasma has been developed and validated by Hedeland et al. [24]. Structures of these molecules and suggested fragmentations are given in Fig. 2. The spectra of both analytes and the internal standard had their most intense signal at *m/z* 165, which was chosen for SRM. The *m/z* 165 signal is presumably due to charge site initiated fragmentation of a relatively weak C–N bond (bond energy 293 kJ mol^{-1}) with hydrogen atom transfer to form a shortened chain ending in $–CH_2=CH_2$. The samples were therefore analysed by LC–ESI-MS/MS in the SRM mode using a deuterated internal standard. The stationary phase used for the chiral separation was Chiral-AGP(R). The enantiomers of verapamil were selectively detected from those of norverapamil by MS due to their different molecular masses although there was chromatographic co-elution. The selectivity of the method towards the metabolites D617, D620 and PR23 was also tested. Lower LODs for the target analytes using this methodology compared to earlier published methods based on fluorescence detection were obtained. The enantiomers of verapamil and norverapamil could be quantified at levels down to 50 pg and 60 pg/500 μL plasma sample,

respectively, with the relative standard deviation (R.S.D.) in the range 3.6–7.8%. This method was successfully applied to an in vivo intestinal absorption and bioavailability study in humans.

A sensitive reduction peak of verapamil has been obtained by adsorptive stripping voltammetry (AdSV) in 0.01 M phosphate (pH 7.4) at an accumulation time of 30 s [25]. The peak potential was −1.81 V (versus Ag/AgCl) and presumably corresponds to reduction of the nitrile bond. The peak current was found directly proportional to the concentration of verapamil (10^{-8} to 10^{-6} M) with a particularly low LOD of 5×10^{-10} M (0.2 ng mL^{-1}). The within day R.S.D. at the 10^{-7} M level was 1.8%. The application of the method to the analysis of urine and pharmaceutical formulations was described. The LOD suggests that AdSV could be used in TDM but the method could not discriminate between verapamil and those metabolites that possess the nitrile bond. The method was found simple with no extraction, rapid, sensitive and reproducible.

There have been no recent papers in the Web of Knowledge database concerning the application of GLC–MS and CE–ESI-MS to the determination of verapamil and its metabolites.

2.3. Lignocaine/lidocaine

Lignocaine/lidocaine has the chain –NH–CO–CH_2–$N(C_2H_5)_2$ attached to a xylene ring (Fig. 3) and its ESI-MS^n behaviour [22] was found somewhat similar to its EI-MS behaviour. Using ESI-MS^n it appeared that at least two methylene groups were required adjacent to the end N atom for fission of the C–N bond and loss of $HN(C_2H_5)_2$ as happens for phenothiazines. Instead in-source fragmentation and MS^2 gave rise to a signal at *m/z* 86 corresponding to $CH_2{=}N^+(C_2H_5)_2$. Application of MS^3 resulted in a signal at *m/z* 58 corresponding to $CH_2{=}N^+H(C_2H_5)$. It should be noted that the signal at *m/z* 86 was observed for chlorpromazine using the same instrumentation but corresponded to a different quaternary nitrogen ion. EI of lignocaine/lidocaine also gave signals at *m/z* 86 and 58 [23]. The amide bond in lignocaine appears not to be broken in these MS^n studies unlike in the break-up of peptides to their constituent amino acids using such MS technology and this is probably due to the amide bond in lignocaine being stabilized by conjugation to the xylene ring.

There have been no recent papers in the Web of Knowledge database concerning the application of LC–ESI-MS to the determination of lignocaine/lidocaine and its metabolites.

2.4. Amphetamines

Amphetamine, C_6H_5–CH_2–$CH(CH_3)NH_2$, (Fig. 3) has shown an $[M+H]^+$ ion at *m/z* 136.2 with minor signals at *m/z* 119.2, 91.4 and 74.2 on application of ESI-MS^n [22]. The *m/z* 119.2 signal corresponded to NH_3 loss $\{\Delta H_f^\circ = -46.1\ \text{kJ mol}^{-1}$ for $NH_3(g)\}$, (Table 2), by charge site initiated fragmentation with H atom transfer resulting in the corresponding alkene. In keeping with this observation, QToF-MS/MS has assigned a elemental formula to an *m/z* 119 signal of C_9H_{11} [22]. $CH_3CH{=}CH_2$ was not lost from the deaminated ion of amphetamine although the signal at *m/z* 91.4 could have corresponded to loss of ethene (28 U).

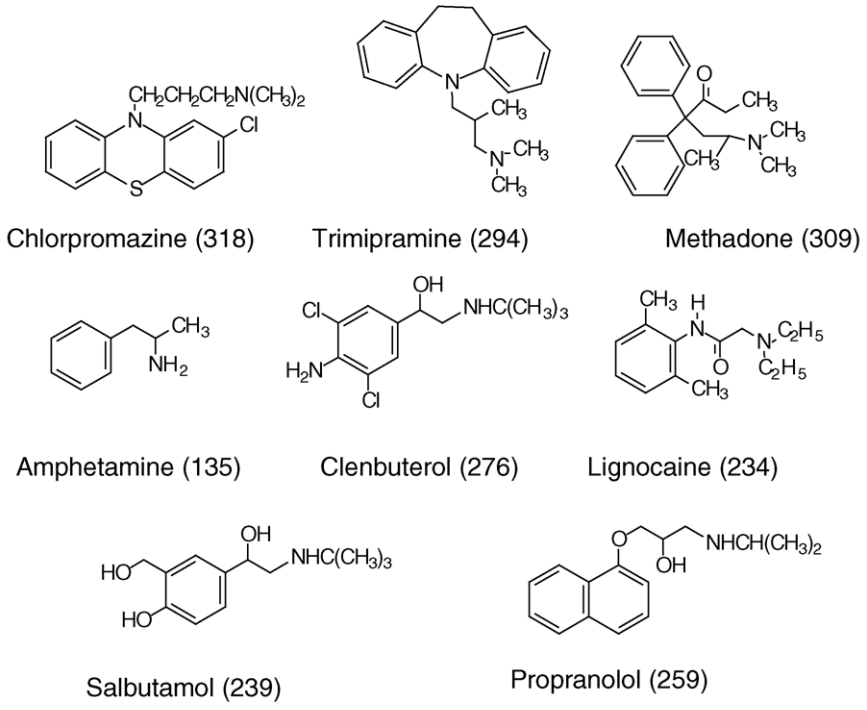

Fig. 3. Names, structures and molecular masses of studied drug compounds with amine-containing side chains [22].

MS^2 and MS^3 gave single signals at *m/z* 118.9 and 91.2, respectively. Such molecules ending with a primary nitrogen atom in its side chain, e.g., amphetamine, lose the amine as ammonia as stated in rules of fragmentation using ion trap techniques [22]. The EI-MS of amphetamine is somewhat different giving a base peak at *m/z* 44 and a small signal at 91 [23].

A procedure based on LC–ESI-MS has been described for determination of amphetamine, methamphetamine, and methylendioxy derivatives in meconium, using 3,4-methylendioxypropylamphetamine as the internal standard [26]. The analytes were initially extracted from the matrix by 17 mM methanolic HCl. Subsequently, SPE with Bondelut Certify columns was applied. Chromatography was performed on a C_{18} reversed-phase column using a linear gradient of 10 mM ammonium bicarbonate, pH 9.0–methanol as a mobile phase. Analytes were determined in SRM mode using ESI-MS. The method was validated in the range 0.005–1.00 μg/g using 1 g of meconium per assay. Mean recoveries ranged between 61.1 and 87.2% for the different analytes. The LOQs were 0.005 μg/g meconium for amphetamine, methamphetamine and 4-hydroxy-3-methoxymethamphetamine and 0.004 μg/g meconium for 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, 3,4-methylenedioxyethylamphetamine, and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine. The method was applied to analysis of meconium in newborns to assess eventual fetal exposure to amphetamine derivatives.

The enantioseparation of nine amphetamine derivatives was studied by comparing two different chiral stationary phases, macrocyclic antibiotic vancomycin and native β-cyclodextrin (β-CD) [27]. Effects of 46 eluent compositions on enantioseparation in reversed-phase and polar organic phase modes were investigated. β-CD was found to be more suitable to phenethylamines in general. An eluent system capable of separating the enantiomers of all phenethylamines in one run was developed. The suitability of the eluent systems to ESI was discussed and methods using a tandem mass spectrometric detection were developed. The suitability of chiral LC–ESI-MS/MS was tested with 14 seized drug samples. The results were in agreement with conventional non-chiral methods. Repeatability of the methods was good and LODs were 25–100 ng mL^{-1} for most compounds. Reversed-polarity CE–ESI-MS and CE–ESI-MS/MS has also been utilised for the simultaneous chiral separation of nine amphetamine stimulants including DL-amphetamine, DL-methamphetamine, DL-methylenedioxyamphetamine, DL-methylenedioxymethamphetamine and DL-methylenedioxyethylamphetamine using highly sulphated γ-cyclodextrin [SU(XIII)-γ-CD] as a chiral selector [29] with the drug–CD complex being dissociated at the ESI interface so that only the drug molecules went into the MS detector.

An ion-pair LC–ESI-MS method with in vivo microdialysis for the determination of free-form amphetamine in rat brain has been developed [28]. A microdialysis probe was surgically implanted into the striatum of the rat and artificial cerebrospinal fluid (aCSF) was used as the perfusion medium. Samples were collected and then analyzed off-line by LC–ESI-MS. A reversed phase C_{18} column was employed for LC separation. Trifluoroacetic acid (TFA) was added in the mobile phase (acetonitrile–water, 10:90, v/v) as an ion-pair reagent. Post-column addition of volatile organic acid was utilised to minimise the TFA signal suppression effect on ESI-MS detection. More than six-fold enhancement of the ESI-MS response was achieved by the post-column addition of propionic acid. Calibration plot linearity (0.01–1.00 μg mL^{-1}, $R^2 = 0.99$) and the LOD (0.002 μg mL^{-1}) were determined. Good precision and accuracy were also obtained. The applicability of this newly developed method was demonstrated by continuous monitoring of amphetamine concentrations in rat brain after a single 3.0 mg/kg i.p. administration.

The analysis of drugs in hair is very topical and is the matrix for some recent LC–ESI-MS and GLC–MS analyses. Attention has to be paid to the decontamination of the hair surface to distinguish between exogenous and endogenous components. 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 aside, however, the attractiveness of the technique is beyond question as far as building up drug profiles and time-histories of abuse of controlled substances are concerned. Following decontamination of the surface, a wide variety of extraction procedures have been employed for drug analysis. These include acid or base treatment followed by solvent extraction, sonication in solvent at elevated temperature, pulverisation of the hair sample followed by liquid–liquid extraction (LLE) and SPE for further purification and Soxhlet extraction. Amphetamine analysis in hair samples by LC–ESI-MS has not appeared in the Web of Knowledge database literature 2004–2005 but a headspace solid-phase microextraction and gas liquid chromatography–mass spectrometry (HS-SPME-GLC–MS) procedure has been published for the simultaneous detection of cocaine, amphetamine (A), methamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), methylenedioxyethamphetamine (MDE), *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB), ketamine and methadone in human hair [30]. Hair was washed with water and acetone in an ultrasonic bath. A short acid extraction with 1 M hydrochloric acid was needed with the fiber being exposed to a 5 min absorption at 90 °C and thermal desorption was performed at 250 °C for 3 min. The procedure was simple, rapid, required small quantities of sample and no derivatisation. Good linearity was obtained over the 0.1–20.0 ng mg^{-1} range for the target compounds. LODs were 0.7 ng mg^{-1} of hair for the majority of substances. The intra-day precision ranged between 7 and 20%. This paper also dealt with the preliminary application to hair samples obtained on a voluntary basis from 183 young people (138 males and 45 females) in the Rome area.

2.5. Methadone

Methadone (Fig. 3), in common with trimipramine in the following subsection, loses the amine $HN(CH_3)_2$ followed by the alkene $CH_2{=}CHCH_3$ in-source with signals at *m/z* values 265.5 and 223.5, respectively, supported by QToF-MS/MS as $C_{19}H_{21}O$ and $C_{16}H_{15}O$ [22]. The former signal was again observed at MS^2 and the latter at MS^3. At MS^3, the signal at *m/z* 265.5 gave a signal at *m/z* 247.1 which corresponded to a dehydration process ($C_{19}H_{19}$ using QToF-MS/MS) [22]. The EI-MS behaviour is substantially different in that a base peak of *m/z* 72 is observed with a variety of small signals [23]. The base peak is due to fragmentation of the amine-containing side chain giving $CH_3CH{=}N^+(CH_3)_2$.

A high-throughput method for the quantitative determination of methadone enantiomers in human plasma has been developed and validated by LC–ESI-MS/MS [31]. The effects of pH and of types and concentrations of mobile-phase modifiers on the enantioselectivity of (*R*)- and (*S*)-methadone were investigated on a Chiral-AGP column. A baseline separation of the enantiomers was achieved with a retention time of less than 5 min. Ionisation suppression and other matrix effects were evaluated. Morphine, cocaine, 6-monoacetylmorphine, benzoylecgonine and ecgonine methyl ester did not interfere with the performance of the assay. The specificity, linearity, intra- and inter-assay precision and accuracy, and extraction recovery were fully evaluated. The method showed excellent reproducibility (overall coefficient of variance <8%) and accuracy (overall bias <2.7%) with a broad linear range. The enantiomers were stable in human plasma after five freeze–thaw cycles, under bench-top storage at room temperature (RT) for 6 h, in the extract reconstitution solution at RT for 17 h, and in processed-extracts stored at RT for 142 h. This validated LC–ESI-MS/MS assay offered high-throughput and improved specificity, sensitivity, linear range and ruggedness over previously published methods and has been successfully applied to the analysis of clinical samples.

An alternative CE–MS assay for methadone enantiomers has been published by Rudaz et al. [32] using highly sulfated γ-cyclodextrin (HS γ-CD) and a headspace solid-phase microextraction and gas liquid chromatography–mass spectrometry (HS-SPME-GLC–MS) procedure has also appeared for the simultaneous detection of cocaine, amphetamines, *N*-methyl-1-(1,3-benzodioxol-5-yl)-2-butanamine (MBDB), ketamine and methadone in human hair [30]. This latter publication has been referred to in the last subsection on amphetamines.

Ion-associate complexes of methadone and other drugs with $[Cd(SCN)_4]^{2-}$ and $[Zn(SCN)_4]^{2-}$ have been precipitated and the excess unreacted cadmium or zinc complex determined using atomic emission and atomic absorption spectrometry for the determination of the these drugs in pure solutions and in pharmaceutical preparations [33]. The drugs could be determined in the range 6.9–69.18 $\mu g\,mL^{-1}$.

2.6. Tricyclics

Trimipramine (Fig. 3) has an MS^n behaviour [22] which is somewhat similar to that of chlorpromazine as discussed under Section 2.1.

There have been no 2004–2005 papers in the Web of Knowledge database on the application of LC–ESI-MS, CE–ESI-MS, GLC–MS and stripping voltammetry to the determination of such tricyclics.

2.7. Clenbuterol and salbutamol

Clenbuterol, containing a carbon chain of structure $–CH(OH)–CH_2–NHC(CH_3)_3$, (Fig. 3), underwent ESI in the positive ion mode to give an $[M+H]^+$ species at a signal value of *m/z* 277.2 with the proton being associated with the aliphatic nitrogen lone pair [22]. Furthermore, two additional signals were observed in this MS mode at *m/z* values 259.4 and 203.4 corresponding to H_2O loss and further loss of $(CH_3)_2C{=}CH_2$ to give a modified chain $–CH{=}CH–NH_3^+$ which is resonance stabilised by conjugation to the substituted benzene ring. The $[M+H]^+$ species lost H_2O at the MS^2 stage to give a signal at *m/z* 258.9 followed by the loss of 56 U at MS^2 corresponding to charge site initiated fragmentation of the $NH–C(CH_3)_3$ bond to ultimately release the neutral molecule $(CH_3)_2C{=}CH_2$ and a signal at *m/z* 203.0. Application of MS^3 to the signal at *m/z* 258.9 yielded a signal at *m/z* 202.9, one at *m/z* 167.1 and one at *m/z* 132.1. The *m/z* 167.1 signal appeared to correspond to removal of HCl. The loss of HCl was regarded as energetically favourable in that for the gaseous state $\Delta H_f^\circ = -92.3\,kJ\,mol^{-1}$. The application of QToF-MS/MS to clenbuterol gave *m/z* signals at 259.0768, 203.0133, 167.0348 and 132.0638 which corresponded to elemental analyses of $C_{12}H_{17}Cl_2N_2$, $C_8H_9Cl_2N_2$, $C_8H_8ClN_2$ and $C_8H_8N_2$, respectively, thus supporting the fragmentation pattern proposed following the above MS^n studies [22]. Pihlainen et al. [34] have also reported on the ESI-MS/MS behaviour of clenbuterol confirming the above fragmentation pathway. EI of clenbuterol [23] follows a substantially different fragmentation pattern with the major ions being found at low *m/z* values 127, 86, 57 and 30. The base peak at *m/z* 86 is due to the ion $(CH_3)_3C–NH^+{=}CH_2$ due to fragmentation of the amine-containing side chain.

An LC–ESI-MS/MS method has been developed for the simultaneous determination of the β-agonists clenbuterol, salbutamol, and cimaterol in bovine retina [35]. The tissue was homogenised in alkaline buffer and spiked to give 10, 15 and 20 ng/g each of the three analytes together with the internal standards D_6-salbutamol and D_6-clenbuterol. The mixture was incubated with protease enzyme to release any protein-bound analytes and then made alkaline before extraction with isobutanol. The extract was dissolved in water and transferred to a clenbuterol immunoaffinity column. After washing, the analytes were eluted and analysed by LC–ESI-MS/MS using a C_{18} column with acetic acid–methanol as the mobile phase. No interferences were observed from the

spiked retina extract in the various SRM modes. Average recoveries for clenbuterol, salbutamol, and cimaterol were 94, 85 and 87% with coefficients of variation of 9.4, 9.9 and 8.6%, respectively. A correlation coefficient of $R^2 = 0.9999$ was obtained for all analytes. The LODs for clenbuterol, salbutamol and cimaterol, determined from three times the standard deviation of seven replicates of the lowest spike, were 2.5, 3.5 and 2.0 ng/g. Garcia et al. [36] have published an alternative GLC–MS assay for the determination of clenbuterol in several biological matrices connected with the 2002/657/EC European Directive.

The related β-agonist, salbutamol, with identical carbon chain but with different substituents in the benzene ring (Fig. 3), gave rise to signals at *m/z* 240.8, 222.5, 166.3 and 148.4 when fragmentation was achieved in-source using ESI-MS^n [22]. This again corresponded to $[M + H]^+$, loss of H_2O, loss of the *t*-butyl group as 2-methylpropene and further loss of H_2O. These signals were also observed at MS^2 and MS^3. The MS^3 signal at *m/z* 148.0 corresponded to H_2O loss from the second aliphatic OH group. Selection of the 166.0 signal at MS^4 gave rise to a single signal at 148.0. The application of QToF-MS/MS to salbutamol gave *m/z* signals at 222.1571, 166.0829 and 148.0692 corresponding to elemental analyses of $C_{13}H_{20}NO_2$, $C_9H_{12}NO_2$ and $C_9H_{10}NO$, respectively, which supported the fragmentation pattern proposed following the MS^n studies [22]. Pihlainen et al. [34] have also reported on the ESI-MS/MS behaviour of salbutamol confirming the above fragmentation pathway.

An LC–ESI-MS/MS method has been developed for the simultaneous determination of the β-agonists clenbuterol, salbutamol and cimaterol in bovine retina [35]. This method has been discussed under clenbuterol. A rival method for the quantification of free salbutamol in human urine has been described using GLC–MS [37]. Sample clean up was performed using SPE on a mixed phase extraction column. Derivatisation was performed with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA). The method was found to be suitable for use in doping detection where a cut-off limit of 1 μg salbutamol/mL urine is set by the International Olympic Committee (IOC) and approved by the World Anti-Doping Agency (WADA). In addition, salbutamol, diphenhydramine, and nortriptyline have been detected in the nmol L^{-1} range using CE–ESI-MS [38].

2.8. Propranolol

Propranolol, with a structure that involves the chain $-O-CH_2-CH(OH)-CH_2-NH-CH(CH_3)_2$, (Fig. 3), gave an $[M + H]^+$ ion at *m/z* 260.6 in the MS mode and no other signals of intensity greater than 5% relative abundance [22]. At MS^2 signals were observed at 242.1 (loss of H_2O), 218.1 (loss of $CH_3CH{=}CH_2$ from $[M + H]^+$), a base peak at 183.1 (loss of $NH_2-CH(CH_3)_2$ from the dehydrated species), a signal at *m/z* 157.1 (loss of C_2H_2 from the deaminated and dehydrated species) and a signal at *m/z* 116.1 which suggested C–O bond fission to give the ion $CH_3CH(OH)CH_2NH^+{=}C(CH_3)_2$. The application of QToF-MS/MS to propranolol gave *m/z* signals at 218.1158, 183.0776, 157.0604, 155.0803 and 116.1016 which corresponded to elemental analyses of $C_{13}H_{16}NO_2$, $C_{13}H_{11}O$, $C_{11}H_9O$, $C_{11}H_7O$ and $C_6H_{14}NO$, respectively, which supported the fragmentation pattern proposed following the MS^n studies [22]. Other workers have also observed major ions at *m/z* 183 and 116 using ESI and a triple quadrupole mass spectrometer [39].

Depuis et al. [40] have developed and validated an LC–ESI-MS method using single quadrupole MS for the identification and determination of propranolol and two other β-blockers in biofluids and solid tissues. Fragmentation was achieved in-source using the positive ion mode with the protonated molecular ion of propranolol at *m/z* 260.2 at an orifice voltage of 20 V being used for quantitation and ions *m/z* 116.0 and 183.0 at an orifice voltage of 50 V being used for confirmation. The LOQs were 50 ng/g in tissues, 50 μg/L in blood and urine and 10 μg/L in vitreous humor.

Wu et al. [41] have studied porcine FAD-containing monooxygenase (FMO) that metabolises lidocaine, bupivacaine and propranolol in vitro and found that FMO metabolised lidocaine, bupivacaine and propranolol. The metabolic products of FMO were separated by LC and analyzed by GLC–MS and were found to be the *N*-oxides and *N*-hydroxylamines.

A study of the electrochemical oxidation of propranolol [42] has revealed a well-defined adsorptive anodic peak was obtained at 0.918 V (versus Ag/AgCl) at pH 2.0. A linear calibration range of 6.0×10^{-7}–5.0×10^{-5} mol L^{-1} and an LOD of 2.0×10^{-7} mol L^{-1} were found using AdSV and the differential pulse mode. The proposed voltammetric approach was applied to the determination of the drug in tablet dosage form. The results were statistically consistent with those obtained with the reference UV method.

2.9. Fluoxetine

Yu et al. [43] have investigated enzymatic tissue digestion as an alternative sample preparation approach for quantitative analysis using LC–MS/MS. Enzymatic digestion is commonly used for tissue dissociation and cell harvesting and offers the advantages of unattended sample preparation, potential automation, and low cost. Two different enzymes (collagenase and proteinase *K*) that are known to degrade connective tissues to allow tissue dissolution were chosen for evaluation, employing well-known antidepressants desipramine and fluoxetine as test compounds in dog and rat brain tissue. Comparison between enzymatic digestion and conventional homogenization tissue preparation was performed, including investigation of matrix ionisation suppression of both methods using a postcolumn infusion system. Results showed that enzymatic digestion had extraction efficiency comparable to homogenisation. Matrix ionisation suppression was not observed for either the test compounds evaluated or the sample extraction method. Test compound levels of incurred tissue samples prepared by enzymatic

digestion were in good agreement with the values obtained by the conventional homogenization tissue preparation, indicating that enzymatic digestion is an appropriate tissue sample preparation method. Initial losses of CH_3NH_2 and $CH_2{=}CH_2$ on fragmentation after ESI are likely bearing in mind how other drugs with amine-containing side chains such as phenothiazines, discussed in Section 2.1, behave under these conditions.

Lamas et al. [44] have published a rival method for fluoxetine assay using solid-phase microextraction-GLC–MS for this and other selective serotonin reuptake inhibitors. Some of the analytes were not efficiently extracted as underivatised compounds and so, an in situ acetylation step was introduced in the sample preparation procedure. The performance of this method was evaluated, showing good linearity and precision. The LODs were in the sub-ng/mL region.

2.10. Ranitidine

Hakala et al. [39] have observed major product ions at *m*/*z* 176 and 270 from the protonated precursor ion at *m*/*z* 315 using ESI with a triple quadrupole mass spectrometer. The loss of 45 U corresponds to charge site initiated fragmentation of the C–N bond at the end of the chain releasing the neutral amine $(CH_3)_2NH$.

A simple and rapid LC–MS/MS method for simultaneous quantification of a mixture of the antineoplastic agent doxorubicin (Dox) and selected emesis suppressants, namely, lorazepam (Lor), metoclopramide (Met), ondansetron (Ond), and ranitidine (Ran), has been developed [45]. 4-Phenyl-4-aminobutanoic acid was used as the internal standard. The analytes were separated on an Xterra(TM) MS, C-18, 2.5 μm (2.1 mm × 30 mm) column using 70% aqueous methanol containing 0.1% formic acid. The investigated compounds were detected by MS/MS using the positive electrospray ionisation mode. The transition at *m*/*z* 315–176 was selected for Ran assay in the simultaneous quantitation of Dox, Lot, Met, Ond and Ran. Calibration curves were constructed over the concentration ranges 10–200 ng mL^{-1} (Dox, Lor), 8–80 ng mL^{-1} (Ond), Met and Ran (1–10 ng mL^{-1}). The correlation coefficient ranged from 0.9953 to 0.9998 with an LOD of 1 ng mL^{-1} for all analytes. The developed LC–MS/MS method quantitatively recovered doxorubicin and the co-admixed compounds in the range 98.1–103.5%. The data suggest the utility of the developed LC–MS/MS procedure for simultaneous routine analysis of mixtures of Dox and the emesis suppressants, Lor, Met, Ond and Ran in i.v. infusions in clinical situations.

2.11. Indole alkaloids

5-Methoxy-*N*,*N*-diisopropyltryptamine (5-MeO-DIPT), a new psychoactive tryptamine derivative, has been synthesised by the Speeter and Anthony procedure [46]. This synthetic route was characterised by ESI-MS/MS, ESI-TOF-MS and NMR. Side products were identified as 3-(2-*N*,*N*-diisopropylamino-ethyl)-1*H*-indol-5-ol, 2-*N*,*N*-diisopropylamino-1-(5-methoxy-1*H*-indol-3-yl)-ethanol, 2-(5-methoxy-1*H*-indol-3-yl)-ethanol and 2-*N*,*N*-diisopropylamino-1-(5-methoxy-1*H*-indol-3-yl)-ethanone. This identification of such key impurities and their MS behaviour made it possible to determine compound-specific ion transitions that could be used for screening purposes. Taking 2-*N*,*N*-diisopropylamino-1-(5-methoxy-1*H*-indol-3-yl)-ethanone as an example, its $[M+H]^+$ ion gave specific *m*/*z* signals at 247, 229 and 114 on application of MS/MS using a collision energy of −17.5 eV with the *m*/*z* 247 signal being due to loss of $H_3C{-}CH{=}CH_2$ from the indole side chain. Thus, the use of MRM studies would allow the unambiguous identification of a synthetic route and the exact quantification of impurities. This is of particular importance in connection with clinical and forensic investigations.

The sensitive determination of psilocin (4-hydroxy-*N*,*N*-dimethyltryptamine) has been carried out RP-HPLC coupled with fluorimetry (FL) and ESI-MS [47]. Psilocin and bufotenine, used as an internal standard (IS), were labelled with excess amounts of 4-(*N*,*N*-dimethylaminosulfonyl)-7-(2-chloroformylpyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-Pro-COCl) at 60 °C for 10 min in the presence of pyridine as the scavenger of HCl produced in the reaction. The resulting derivatives were separated by a Mightysil RP-C_{18} GP column (150 mm × 4.6 mm, i.d., 3 μm) with an acidic mobile-phase containing 0.1% trifluoroacetic acid and detected at 560 nm (excitation at 440 nm). Under the conditions for derivatisation, separation and detection, adherence to linearity of the calibration curve of psilocin was observed by the LC-FL method. On the other hand, the derivative, again separated by the Mightysil RP-C_{18} GP column (100 mm × 2.0 mm, i.d., 3 μm) using 50 mM ammonium acetate: acetonitrile (73:27, v/v) mobile phase, was also determined by LC–ESI-MS. The mass spectrometer was operated in the SIM mode for the protonated-molecular ion, $[M+H]^+$ (*m*/*z* = 527). When this ion was used as the precursor ion for MS/MS analysis, the product ions, i.e., at *m*/*z* = 482, 295 and 205, were identified for both derivatives. These ions would appear to be due to the cleavage of dimethylamino, carboxylic acid ester and DBD-Pro moieties. The calibration curve in the SIM mode was linear in the range of 0.16–4.08 ng psilocin, a similar range to the LC-FL method. The coefficient of variation (CV) was 5.15% (0.16 ng injection, *n* = 6). The LOQ was 0.64 ng/mg dried mushroom. The amounts of psilocin in six "magic" mushrooms using LC–MS were lower than 12.67 ng/mg samples. The developed method was also successfully applied to the determination of psilocin in rat plasma after a single i.p. administration of psilocybin.

3. Drugs with N-containing saturated ring structures

3.1. Morphine and codeine

A specific and simultaneous assay of morphine, morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-

G) in monkey and dog plasma has been developed [48]. These methods were based on rapid isolation using SPE and then LC–ESI-MS/MS. Analytes were separated on a semi-micro ODS column in acetonitrile–formic (or acetic) acid mixed solution. The calibration linearity of morphine, M-3-G and M-6-G was confirmed in the concentration ranges of 0.5–50, 25–2500 and 2.5–250 ng/mL in monkey plasma, 0.5–100, 25–5000 and 2.5–500 ng/mL in dog plasma, respectively. The precision of this assay method, expressed as CV, was less than 15% over the entire concentration range with adequate assay accuracy. Therefore, the LC–ESI-MS/MS method was found useful for the determination of morphine, M-3-G and M-6-G with sufficient sensitivity and specificity in pharmacokinetic studies.

A rival GLC–MS procedure has been developed for the detection of opiates in whole blood and the quantitative determination of morphine and codeine as their propionic esters [49]. The LOD for morphine and codeine in blood was found to be 0.02 μg/mL and the linear calibration range 0.05–2.0 μg/mL, somewhat less sensitive than the LC–ESI-MS method [48]. The maximum intra-assay relative errors were 15.6 and 11.2% for concentrations of 0.05 and 1.0 μg/mL, respectively, and the inter-assay relative errors were no higher than 8 and 3% for concentrations of 0.05 and 1.0 μg/mL, respectively. The procedure was recommended for forensic analysis in cases of opiate poisoning.

CE–MS^n and computer simulation of fragmentation have been demonstrated to be effective tools to detect and identify phases I and II metabolites of oxycodone (OCOD) in human urine [50]. OCOD is a strong analgesic used for the management of moderate to severe mainly postoperative or cancer-related pain whose metabolism in man is largely unknown. Using an aqueous pH 9 ammonium acetate buffer and CE–MS^n ($n \leq 5$), OCOD and its phase I metabolites produced by *O*-demethylation, *N*-demethylation, 6-ketoreduction and *N*-oxidation (such as oxymorphone, noroxycodone, noroxymorphone, 6-oxycodol, nor-6-oxycodol, oxycodone-*N*-oxide and 6-oxycodol-*N*-oxide) and phase II conjugates with glucuronic acid of several of these compounds were detected in alkaline solid-phase extracts of a patient urine that was collected during a pharmacotherapy episode with daily ingestion of 240–320 mg of OCOD chloride. The data for three known OCOD metabolites for which the standards had to be synthesised in-house, 6-oxycodol, nor-6-oxycodol and oxycodone-*N*-oxide, were employed to identify two new metabolites, the *N*-oxidized derivative of 6-oxycodol and an *O*-glucuronide of this compound. CE–MS^n and computer simulation of fragmentation also led to the identification of the *N*-glucuronide of noroxymorphone, another novel OCOD metabolite for which no standard compound or mass spectra library data were available.

3.2. Cocaine

The $[M+H]^+$ ion of cocaine at *m*/*z* 304.1 underwent in-source fragmentation to give a signal at *m*/*z* 182.1, a signal that is also observed at MS^2 on application of ESI-MS^n for this drug molecule [51]. This was due to the loss of the benzoic acid substituent as a neutral molecule, leaving a secondary carbonium ion and was supported by QToF-MS/MS data with elemental analysis data of $C_{10}H_{16}NO_2$ for a signal at 182.1195. MS^3 and MS^4 gave major signals at *m*/*z* 150.0 and 122.2, which corresponded to successive loss of CH_3OH and CO from the methyl ester substituent. These substituent losses were also observed in EI, together with a variety of other low mass signals and a relatively intense signal at *m*/*z* 82 [23].

LC–ESI-MS has not been applied to the analysis of cocaine in the period 2004–2005 according to the Web of Knowledge database. However, automated SPE followed by GLC–MS using 2,2,3,3,3-pentafluoro-1-propanol (PFP)/pentafluoropropionic anhydride (PFPA) derivatives has been reported [52] for the simultaneous determination of cocaine (COC) and its metabolites, i.e., benzoylecgonine (BE), norbenzoylecgonine (NBE), ecgonine methyl ester (EME), ecgonine (E), and norcocaine (NCOC), as well as anhydroecgonine methyl ester (AEME) (a unique byproduct of COC smoking), cocaethylene (a molecule formed by the concurrent use of COC and ethanol) and their related metabolites, anhydroecgonine (AE), norcocaethylene (NCE), and ecgonine ethyl ester (EEE). LODs ranged from 0.78 to 12.5 ng/mL and the linear dynamic range for most analytes was 0.78–3200 ng/mL. This method was applied to five aviation fatalities and was proven to be simple, robust and accurate for the simultaneous determination of COC and 11 COC metabolites in postmortem fluids and tissues.

The electrochemical properties of cocaine and its metabolites such as benzoylecgonine, ecgonine, and ecgonine methyl ester have been studied by means of square-wave voltammetry (SWV) at a hanging mercury drop electrode and recommendations made as to the applicability of AdSV for their trace analysis [53].

3.3. Other drugs with nitrogen-containing saturated ring structures

Smyth et al. [51] have carried out ESI-MS^n studies on selected drugs including nicotine, olanzapine, risperidone, narcotine, yohimbine, pethidine, prazosin and sildenafil (Fig. 4). Supported by QToF-MS/MS data, they have shown certain characteristic fragmentations in that functional groups were generally cleaved from the ring systems as neutral molecules such as H_2O, amines, alkenes, esters, carboxylic acids, etc. as expected from their $-\Delta H_f^\circ$ values (Table 2). They stated that when such a nitrogen-containing drug molecule also contained a functional group such as an ester that on liberation as a neutral molecule had a significantly lower ΔH_f° value than that of the corresponding amine then the former was preferentially liberated (Fig. 4). Furthermore, when an aromatic entity was present in a drug molecule together with a nitrogen-containing saturated ring structure

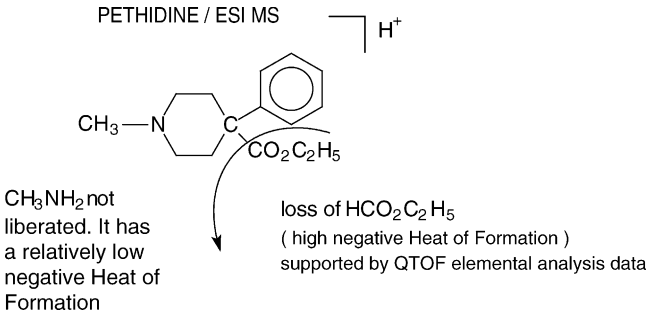

Fig. 4. Fragmentation processes for pethidine using ESI-MS^n [51].

fragmentation occurred to the latter ring with the former being predictably resistant to fragmentation. The data therefore provided useful information on the structure of these compounds and could be used in the characterisation of such drugs and their structurally related metabolites. The authors also reported that the ESI-MS^n data of such compounds could be held in a database and neutral mass losses cross-referenced with such data obtained from unknown analytes which could then be of value in their structural characterisation with respect to those molecules with nitrogen-containing saturated ring structures.

Olanzapine, risperidone, clozapine (CLZ) and quetiapine (QTP) have been widely used in the treatment of schizophrenia and little study has been conducted to determine the four drugs simultaneously by the use of LC–ESI-MS. A sensitive method has been developed for the simultaneous determination of CLZ, OLZ, RIP and QTP in human plasma by LC–ESI-MS [54]. The analytes were extracted twice by ether after samples had been alkalinised. The LC separation of the analytes was performed on a C_{18} column, using water (formic acid: 2.70 mmol/L, ammonium acetate: 10 mmol/L)–acetonitrile (53:47) as mobile phase, with a flow-rate of 0.16 mL/min. The compounds were ionised by ESI and were detected using SIM. The calibration curves were linear in the ranges of 20–1000 ng/mL for CLZ and QTP, 1–50 ng/mL for OLZ and RIP, respectively. The average extraction recoveries for all the four analytes were above 80%. The intra- and inter-day R.S.D. values were less than 15%. The method was found accurate, sensitive and simple for routine TDM measurements and for the study of the pharmacokinetics of the four drugs.

A routine method has been developed for detecting piperidine-containing methylphenidate (Ritalin) use among drug abusers using LC–ESI-MS/MS) [55]. This methodology was designed to replace less reliable and/or more expensive and time-consuming techniques (GLC–MS and ELISA) currently employed in the laboratory and to provide a combined one-step screening and confirmation LC–MS/MS method. Random urine samples sent for drugs of abuse testing, standards, and controls were diluted 1:100 in methanol. Diluted specimens were injected directly into LC–ESI-MS/MS instrumentation. The method utilised SRM to detect both urinary methylphenidate and the more prevalent metabolite, ritalinic acid (RA). Methylphenidate and RA both fragmented with the loss of a positively charged piperidine entity at *m*/*z* 84. There appeared to be little or no sacrifice in sensitivity because the higher dilutions exhibited much less matrix effect. The LOQ for methylphenidate was 100 and 500 nM for RA. Linear calibration curves from 100 to 1000 nM for Ritalin and 500 to 5000 nM for RA were acquired. Precision of analysis of spiked and real samples did not exceed 10% and, at the LOQ, was less than 20%. The performance of the LC–MS/MS method was found superior to GLC–MS or ELISA and it allowed use of a single rapid procedure for both screening and confirmation.

The ESI-MS^n of nicotine and several of its degradation products and their subsequent fragmentations using an ion trap mass spectrometer have been investigated by Smyth et al. [56] for use in establishing the purity of nicotine formulations.

Taylor et al. [57] have described an LC–ESI-MS/MS method for the measurement of nicotine in human plasma. Samples (500 μL) with added D_3-nicotine as an internal standard were treated with a two-step process of ether extraction (6 mL) followed by back-extraction into 0.1% formic acid (50 μL). LC was performed on a phenyl Novapak column with a mobile phase consisting of 50% 10 mM ammonium formate (pH 3.3) and acetonitrile (50:50, v/v). A flow rate of 0.2 mL/min resulted in a total analysis time of 5 minutes per sample. Mass spectrometric detection was by SRM (nicotine *m*/*z* 163.2–130.2; IS *m*/*z* 166.2–87.2). The assay was linear from 0.5 to 100 μg/L ($r > 0.993$, $n = 9$). The accuracy and precision of the method for quality control samples were 87.5–113% and <10.2%, respectively. Interday accuracy and precision at the LOQ (0.5 μg/L) was 113% and 7.2% ($n = 4$). This method was used successfully to measure the pharmacokinetic profiles of subjects involved in the development of an aerosol inhalation drug delivery system.

4. Drugs with N-containing unsaturated ring structures

4.1. 1,4-Benzodiazepines

The ESI-MS^n of 16 pharmacologically significant 1,4-benzodiazepines and their subsequent fragmentation using an ion trap mass spectrometer have been studied by Smyth et al. [58]. The data presented in this paper has provided useful information on the fragmentation processes and can be used in the characterisation of this important class of drugs and their metabolites. Taking the 7-nitro compounds and their metabolites as examples of a particular structural class of 1,4-benzodiazepines, nitrazepam and flunitrazepam underwent ESI by protonation in the positive ion mode. The resulting protonated species $[M + H]^+$ then lost NO_2, (46 U), using the MS^2 mode. This ejection of NO_2 is not energetically advantageous since its ΔH_f° value is +33.2 kJ mol^{-1}. It is therefore assumed that the resulting cation radical must

be relatively stable which should be coupled to the fact that the C–N bond is of relatively low energy (293 kJ mol^{-1}). MS^3 of this cation radical then gave a 29 U loss which could be correlated with the loss of a radical such as COH or the neutral molecule CH_2=NH with subsequent contraction of the seven-membered ring to a six- or five-membered one, respectively. QToF-MS/MS data for flunitrazepam [59] has supported the *m*/*z* signals at 268 and 239 being due to loss of the nitro substituent and the CHO radical, respectively. The EI-MS of flunitrazepam [23] gives major fragment ions at relatively high *m*/*z* values of 294, 285, 266, 255 and 238 and a series of low intensity signals at low *m*/*z* values suggesting that this EI fragmentation for a 1,4-benzodiazepine is somewhat similar in part to that observed using ESI-MS^n and ESI-QtoF-MS/MS techniques. This similarity between EI-MS and ESI-MS^n for such benzodiazepines is in contrast to some of the drugs already discussed in this review such as methadone and clenbuterol.

Some of the metabolites of these 7-nitro-benzodiazepines, e.g., 7-amino-substituted benzodiazepines such as 7-aminonitrazepam and 7-aminoflunitrazepam are resistant to loss of the 7-amino substituent. The former compound lost 28 U at MS^2 which corresponded to loss of CO from its heterocyclic ring and also gave a signal at *m*/*z* 121.2 which corresponded to break-up of its heterocyclic ring system [58]. 7-Aminoflunitrazepam showed a more extensive fragmentation profile with HF being lost at MS^2 to give a resonance stabilised carbocation at *m*/*z* 264.1 [59]. CO was then lost at MS^3 giving a six-membered heterocyclic ring. Twenty-eight units was then lost at MS^4 due probably to loss of a NCH_2 radical followed by loss of 27 U at MS^5 giving a species of *m*/*z* 181.2. QToF-MS/MS data [59] confirmed the fragmentation pathway discussed above.

A rapid, sensitive and specific LC–ESI-MS method to quantify bromazepam in human plasma using diazepam as the IS has been described [60]. The analyte and the IS were extracted from plasma by liquid–liquid extraction using diethyl ether–hexane (80:20, v/v). The extracts were analysed by reversed phase LC–ESI-MS/MS. The method had a chromatographic run time of 5.0 min and a linear calibration curve over the range 5.0–150 ng mL^{-1} ($R^2 > 0.9952$). The LOQ was 5 ng mL^{-1}. This LC–ESI-MS/MS procedure was used to assess the bioequivalence of two bromazepam 6 mg tablet formulations.

Many alternative GLC–MS methods for 1,4-benzodiazepine analysis have been published in the literature. For example, in 2004, Pirnay et al. [61] have published such a method for the simultaneous determination of buprenorphine, flunitrazepam and their metabolites in rat plasma with application to pharmacokinetic studies. Plasma samples were extracted by liquid-liquid extraction using Toxitubes A. Extracted compounds were derivatized with *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), using trimethylchlorosilane (TMCS) as a catalyst. They were then separated by GLC on a crosslinked 5% phenylmethylpolysiloxane analytical column and determined by quadrupole MS operated in the SIM mode. Excellent linearity was found between 0.125 and 5–25 ng/μL plasma for most of the compounds and between 0.025 and 50 ng/μL for 7-aminoflunitrazepam (7-AFNZ). The LOQ was 0.025 ng/μL plasma for 7-AFNZ and 0.125 ng/μL for the four other compounds. A good reproducibility (intra-assay CV = 0.32–11.69%; inter-assay CV = 0.63–9.55%) and accuracy (intra-assay error = 2.58–12.73%; inter-assay error = 0.83–11.07%) were attained. Recoveries were in the range 51–81%. The GLC–MS method was successfully applied to pharmacokinetic studies.

CE–ESI-MS can also be applied to benzodiazepine assay but generally with higher LOD values. For example, McClean et al. [62] have published a paper on the identification and determination of selected 1,4-benzodiazepines(diazepam and its metabolites, desmethyldiazepam, oxazepam and temazepam) by an optimised CE–ESI-MS method. By investigating constituent parts of the capillary electrophoresis-electrospray mass spectrometric interface and optimising their function, a relatively fast and reproducible method was described for the identification and determination of these 1,4-benzodiazepines.

Polarography/stripping voltammetry has also been significantly exploited for benzodiazepine analysis. In 2004, E-Hefnawey et al. [63] have published on the voltammetric behaviour and quantification of the benzodiazepine chlordiazepoxide in bulk form, pharmaceutical formulation and human serum at a mercury electrode. Based on the interfacial adsorptive character of the drug onto the mercury electrode, a validated square-wave adsorptive cathodic stripping (SWAdSV) voltammetric procedure has been described for the trace determination of the drug in bulk form, tablets and human serum. The procedure did not require sample pretreatment or time-consuming extraction or evaporation steps prior to the assay of the drug. The proposed procedure was found to have particularly low LODs (4.4×10^{-10} M and 6.6×10^{-10} M) and LOQs (1.5×10^{-9} M and 2.2×10^{-9} M) in a pharmaceutical formulation and spiked human serum, respectively.

4.2. Zopiclone and zolpidem

Smyth et al. [59] have studied the ESI-MS^n behaviour of the hypnotics, zopiclone, zolpidem, flunitrazepam and their metabolites (Fig. 5) which have shown certain characteristic fragmentations in that functional groups are generally cleaved from the ring systems as neutral molecules such as H_2O, CO, CO_2, NO_2, amines and HF. When an aromatic entity was present in a drug molecule together with a nitrogen-containing saturated ring structure as with zopiclone and its *N*-desmethyl metabolite fragmentation initially occurred at the latter ring with the former being predictably resistant to fragmentation. The structures of fragment ions proposed for ESI-MS^n was supported by ESI-QToF-MS/MS. The application of LC–ESI-MS showed that generally the MS/MS mode gave linear calibration plots down to lower concentrations

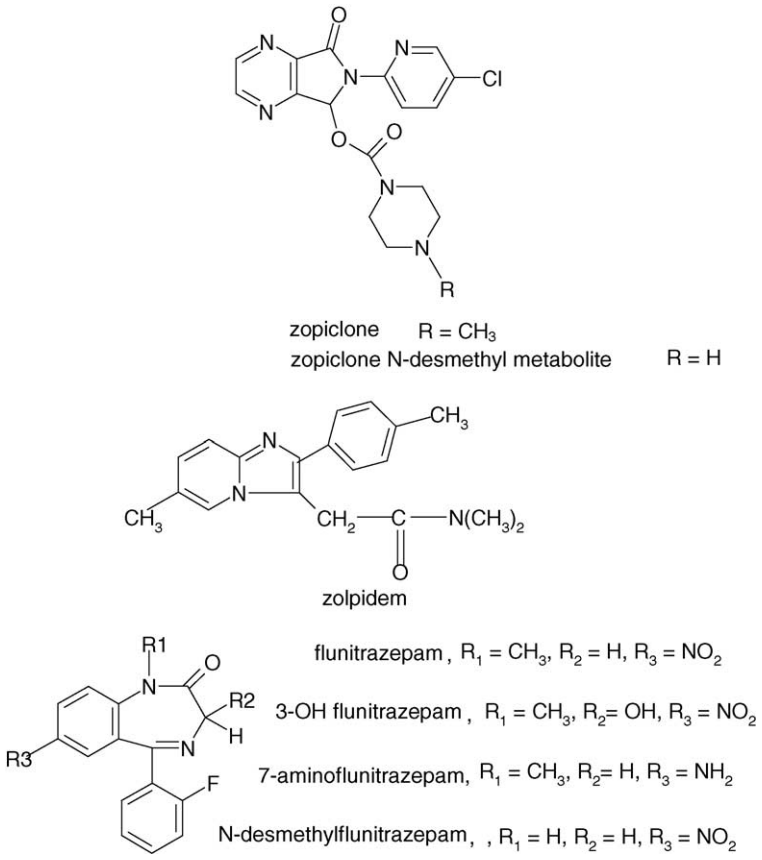

Fig. 5. Molecular structures of zopiclone, zolpidem, flunitrazepam and their metabolites.

than the MS mode with R^2 values of 0.99 or better. LC–ESI-MS of a mixture of the three hypnotics and their metabolites showed baseline resolution and the ability of this technique to identify and quantify a particular hypnotic or its metabolite in question. Zopiclone and its *N*-desmethyl metabolite, however, chromatographed at essentially the same retention time under these conditions.

The saliva matrix is increasingly being used for drug testing to monitor illicit and licit drug use. Saliva is the natural ultrafiltrate of plasma with molecules transported across epithelial membranes. Highly bound drugs are unlikely to cross the cellular membranes therefore saliva testing offers the possibility of direct comparison of unbound, pharmacologically active drug concentrations with the observed effects. Smyth et al. [59] have applied LC–ESI-MS to the determination of a mixture containing 5×10^{-7} mol L^{-1} concentrations of zopiclone, zolpidem, flunitrazepam and their metabolites spiked in 200 μL saliva using SIM of the protonated molecular ions. The analytical method involved acetonitrile being used to precipitate proteins. Recoveries were calculated to be in excess of 90% and the method could be used to monitor low ng/mL concentrations of these hypnotics and their metabolites in saliva.

A validated GLC–MS procedure operating in the SIM mode has been presented [64] for the simultaneous quantitative screening of 51 drugs of abuse or drugs potentially hazardous for traffic safety in serum, plasma or whole blood. Drugs such as zopiclone, zolpidem, cocaine, benzodiazepines, cannabinoids, opioids and others including the internal standard flurazepam were isolated by liquid–liquid extraction followed by derivatisation of the dried extracts by two-step silylation. Intra- and inter-day precisions were within 2.5–21.8 and 6.0–22.5% and R^2 values ranged from 0.9896 to 0.9999. The LOQ for the analytes varied from 2 to 2000 ng/mL and the method was suggested for use in clinical and forensic toxicological analyses.

4.3. Carbadox

A sensitive and selective method using LC–ESI-MS for the determination of carbadox metabolites, quinoxaline-2-carboxylic acid (QCA) and desoxycarbadox (Desoxy-CDX) in swine muscle and liver has been developed [65]. The LC separation was performed on a Cadenza CD-C_{18} column (10 cm × 2 mm, i.d.) with a gradient system of 0.01% acetic acid–acetonitrile as the mobile phase at a flow rate of 0.2 mL/min. Negative ionisation produced the $[M-H]^-$ molecular ion of QCA. On the other hand, the positive mode produced the $[M+H]^+$ ion of Desoxy-CDX. The calibration graphs for QCA and Desoxy-CDX were rectilinear from 0.01 to 0.5 ng with SIM. The drugs were extracted with 0.3% metaphosphoric acid–methanol (7:3) and the extracts

were cleaned up on an Oasis HLB cartridge (60 mg) and by liquid–liquid extraction. The recoveries of QCA and Desoxy-CDX from swine muscle and liver fortified at 2.5 and 5 ng/g were 70.2–86.3%, and the LODs were 1 ng/g for both drugs.

Hutchinson et al. [66] have recently used LC–ESI-MS/MS to confirm carbadox and olaquindox metabolites in porcine liver. A method has been described for the quantitative determination of quinoxaline-2-carboxylic acid and methyl-3-quinoxaline-2-carboxylic acid (MQCA), the metabolites that have been designated as the marker residues for the veterinary drugs, carbadox and olaquindox, respectively, in swine tissue. This method is suitable for use as a confirmatory method under EU National Surveillance Schemes. Porcine liver samples were subjected to protease digestion followed by liquid–liquid extraction. Further clean-up was performed by automated SPE and was followed by a final liquid-liquid extraction step. Analysis was performed using a narrow bore column LC coupled to electrospray MS/MS, operated in positive ion mode. MS/MS product ions were monitored at *m*/*z* 102 and 75 for QCA, *m*/*z* 145 and 102 for MQCA and at *m*/*z* 106 and 152 for the D_4-QCA and D_7-MQCA internal standards, respectively. The method was validated at 3.0, 10, 50 and 150 $\mu g\,kg^{-1}$ for both metabolites. The method performance characteristics-the decision limit (CCalpha) and the detection capability (CCbeta) were determined for QCA at 0.4 and 1.2 $\mu g\,kg^{-1}$, respectively, and for MQCA at 0.7 and 3.6 $\mu g\,kg^{-1}$, respectively.

A rival method using isotope dilution gas chromatography–electron capture negative chemical ionisation mass spectrometry (GC–ECNI-MS) has been developed and validated for the analysis of quinoxaline-2-carboxylic acid in porcine liver [67]. D_4 QCA was added to liver samples which were then deproteinated with 2% metaphosphoric acid in 20% methanol. After sequential extraction with water-saturated ethyl acetate and phosphate buffer, the buffer extracts were subject to SPE clean-up by mixed mode anion-exchange columns. QCA was derivatized with *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) prior to GC–ECNI-MS determination. For unambiguous identification, a second GC–ECNI-MS experiment was performed on suspected positive samples which were derivatised independently with another derivatisation agent, trimethylsilyldiazomethane. Excellent recovery and precision were obtained and the LOQ was 0.7 μg/kg using the LOQ defined as 10 times the standard deviation of the blank signal.

5. Quaternary ammonium drugs

Quaternary ammonium drugs such as anticholinergic agents have been known to be abused in equine sports. A general screening method for this class of drugs in equine urine by LC–MS has not been reported. This paper described an LC–MS/MS method for the simultaneous detection and confirmation of twenty quaternary ammonium drugs at low $ng\,mL^{-1}$ in equine urine after SPE [68]. Quaternary ammonium drugs were extracted from equine urine by SPE and analysed by LC–MS/MS in the positive ESI mode. Separation of twenty quaternary ammonium drugs (the quaternary ammonium ions of edrophonium chloride, pyridostigmine bromide, neostigmine bromide, bretylium tosylate, ipratropium bromide, tubocurarine chloride, *N*-butylscopolammonium bromide, mepenzolate bromide, rocuronium bromide, clidinium bromide, pipenzolate bromide, isopropamide iodide, glycopyrronium bromide, alcuronium chloride, oxyphenonium bromide, propantheline bromide, tridihexethyl chloride, vecuronium bromide, mivacurium chloride and poncuronium bromide) was achieved on an RP column with a mixture of aqueous ammonium formate, (pH 3.0, 10 mM) and acetonitrile as the mobile phase. Detection and confirmation of the twenty quaternary ammonium drugs at concentrations of $5\,ng\,mL^{-1}$ in equine urine could be achieved within 22 min using product-ion scan MS/MS. Negative samples ($n = 30$) of normal post-race equine urine have also been analysed and no matrix interference at the targeted masses and retention times was observed. The method was successfully applied to the analyses of drug-administration samples.

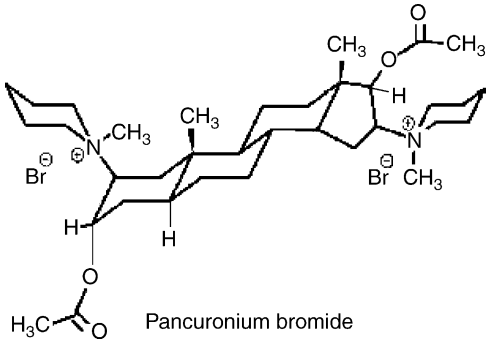

Fig. 6. Structure of pancuronium bromide.

The isolation and detection of pancuronium bromide (Fig. 6) has been developed for aged autopsy samples to identify and confirm this compound in tissue samples [69]. A novel protocol was optimised for the isolation of the target drug in highly decomposed tissues. SPE cartridges containing styrene-divinylbenzene retained this quaternary drug and on elution the semi-purified SPE samples were prescreened by pyrolysis GLC–MS. The identity of the drug was then confirmed by microbore LC–ESI-MS/MS with a triple-quadrupole mass spectrometer. The developed procedures provided a qualitative/semiquantitative basis for the investigation of difficult cases involving overdoses of polar drugs.

6. Conclusions

This review has considered recent studies on the ESI-MS of selected small molecular mass nitrogen-containing

drug molecules, their metabolites, formulation degradation products and process impurities taken from both studies in the author's laboratory and the recent literature using the Web of Knowledge database (http://wos.mimas.ac.uk). The structural classes are drugs with amine-containing side chains, drugs with nitrogen-containing saturated ring structures, drugs with nitrogen-containing unsaturated ring structures and quaternary ammonium drugs. Details have been given on the fragmentations that the different classes of drugs exhibit which will be of use to relevant researchers.

A review of applications for the period 2004–2005, again taken from the Web of Knowledge database, of the technique LC–ESI-MS to the detection and determination of these nitrogen-containing drug molecules in biomatrices, pharmaceutical formulations, etc., has then been made. Analytical information on, for example, sample concentration techniques, LC separation conditions, recoveries from biological media, degradation products and limits of detection have been provided, again of value to those involved in drug analysis. Comparisons, where available, have also been made with rival analytical techniques such as GLC–MS, CE–ESI-MS and SV.

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