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The characterisation of selected drugs with amine-containing side chains using electrospray ionisation and ion trap mass spectrometry and their determination by HPLC–ESI-MS

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Abstract

The electrospray ionisation–ion-trap mass spectrometry (ESI-MS^n) of selected drug compounds with amine-containing side chains has been investigated. Certain characteristic in-source fragmentations have been observed for these molecules. Sequential product ion fragmentation experiments (MS^n) have been performed in order to elucidate the degradation pathways for the $[M + H]^+$ ions and their predominant fragment ions. These MS^n experiments also show certain characteristic fragmentations with respect to the amine-containing side chains. QTOF-MS/MS has been used to support the identity of the proposed fragments. The data presented in this paper therefore provides useful information on the structure of these compounds with amine-containing side chains and can be used in the characterisation of such drugs, their structurally related metabolites and unknown molecules of pharmaceutical significance extracted from animal and plant sources, for example.

Amphetamine, clenbuterol, flurazepam and methadone can be identified and determined in mixtures at low ng/ml concentrations by the application of HPLC–ESI-MS which can also be used for their analysis in saliva samples.

Keywords: Electrospray mass spectrometry; Ion trap fragmentation; Drugs

1. Introduction

Smyth et al. [1] have carried out a study of the ESI-MS^n behaviour of some twelve selected drugs with nitrogen-containing saturated ring structures such as piperidines and piperazines with a view to establishing rules of fragmentation that can be used for characterisation purposes. The 12 selected drugs were pethidine, risperidone, cocaine, narcotine, reserpine, yohimbine, prazosin, sildenafil, olanzapine, morphine, codeine and nicotine. It was found that fragmentation of these drugs with nitrogen-containing saturated ring structures using ESI-MS^n resulted in functional groups being cleaved from the ring systems as neutral molecules such as H_2O, amines, alkenes, esters, carboxylic acids, etc. When an aromatic entity was present in these drug molecules together with the nitrogen-containing saturated ring structure fragmentation occurred to the latter ring with the former being predictably resistant to fragmentation. The structures of fragment ions proposed for ESI-MS^n could be supported by electrospray ionisation–quadrupole-time-of-flight mass spectrometry (ESI–QTOF-MS). The data presented in this paper therefore provided useful information on the structure of these heterocyclic compounds which could be used to characterise unknown drug compounds isolated from natural sources, for example.

A further paper [2] followed on from these studies by investigating the ESI-MS^n and ESI–QTOF-MS/MS behaviour of selected hypnotic drugs and their metabolites again with the intention of establishing rules of fragmentation. In addition, their HPLC–ESI-MS^n behaviour was also studied with

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a view to the identification and quantitation of these drugs in mixtures at ng/ml concentrations and their determination in saliva, a new biological matrix for drug testing to monitor illicit and licit drug use. The drugs chosen were zopiclone and its *N*-desmethyl metabolite, zolpidem, flunitrazepam and its 7-amino, *N*-desmethyl and 3-hydroxy metabolites. These drugs are well known therapeutic agents but are also abused as in date rape situations.

Smyth [3] has reviewed selected publications since 1993 concerning the HPLC–ESI-MS analysis of drugs and their metabolites in order to investigate the generality of such rules of fragmentation. The drugs were chosen according to selected structural classes in which the molecules gave ESI signals primarily as $[M + H]^+$ ions, i.e., drugs with amine-containing side chains, drugs with *N*-containing saturated ring structures, 1,4-benzodiazepines, aminoglycosides, polyethers, thiol-containing heterocycles, sulphonylureas, anthracyclines, penicillins, cephalosporins, nitrocatechols, steroids, macrolides and miscellaneous molecules. Details were given on the fragmentations that these ionic species exhibit in-source, in triple quadrupoles and in ion-traps. Where possible, the molecular structures of the fragments were assigned. The review was principally concerned with the ESI-MS behaviour of these drugs and their metabolites but details were also given of corresponding SPE and HPLC procedures prior to ESI-MS.

This paper follows on from these earlier papers with a study of the ESI-MS^n behaviour of selected drugs with

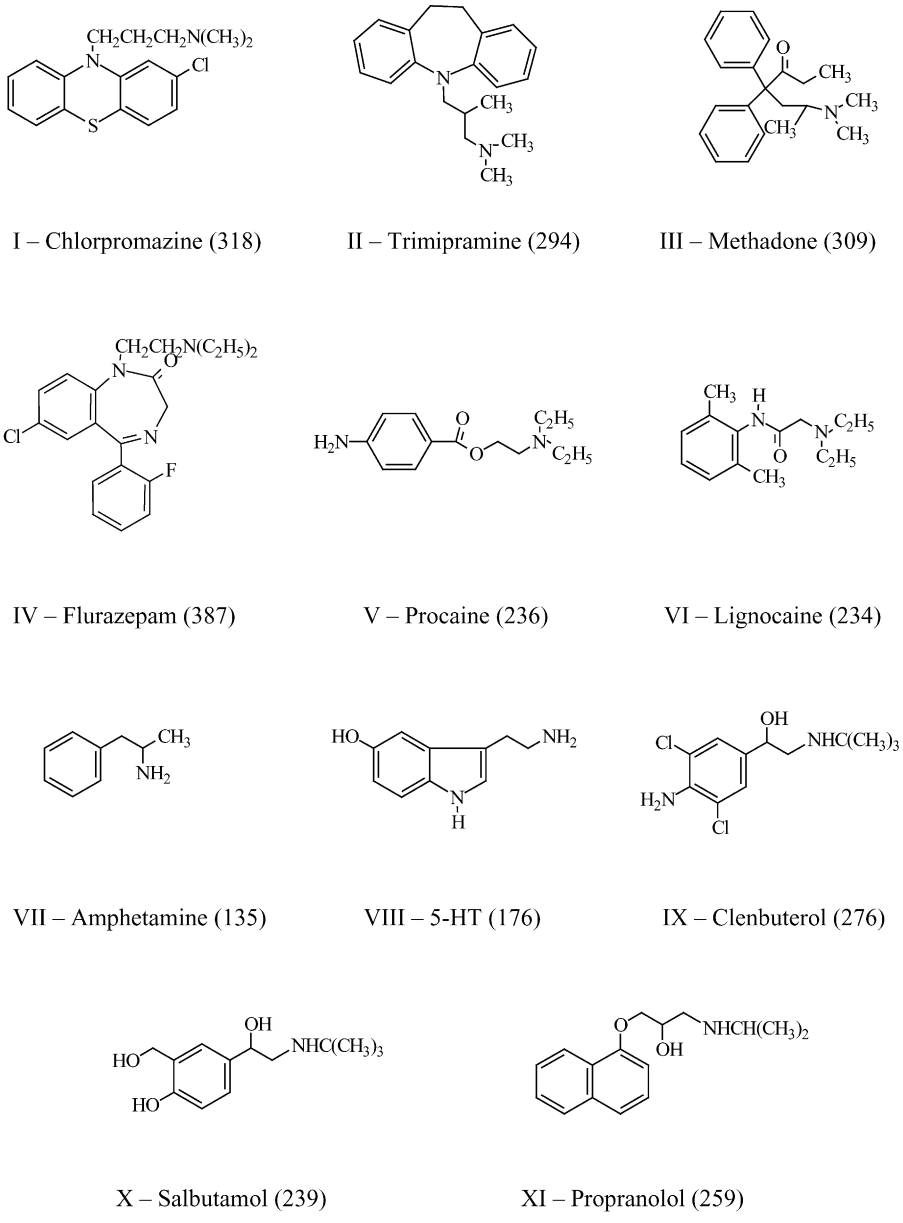

Fig. 1. Names, structures and molecular masses of studied drug compounds.

amine-containing side chains. Drugs with amine-containing side chains are not uncommon as pharmaceuticals as shown for compounds (I) to (XI). The names, structures and the molecular masses of the compounds are given in Fig. 1. Phenothiazine antipsychotics such as chlorpromazine, (I) promethazine, promazine, propionylpromazine and acepromazine, discussed in this paper, all contain a carbon chain linked to the nitrogen atom of the phenothiazine ring system and end in a tertiary nitrogen atom. Tricyclic antidepressants such as trimipramine (II) also have a carbon chain from the nitrogen atom of the tricyclic system ending in a tertiary nitrogen atom. The analgesic, methadone, (III), has also a carbon chain ending in a tertiary nitrogen atom. The sedative, flurazepam, (IV) has a similar chain attached to the $N-1$ atom of the diazepine ring. Procaine (V) is used for anaesthesia and has a side chain of structure $-C(=O)-O-(CH_2)_2-N(C_2H_5)_2$. The local anaesthetic drug lignocaine/lidocaine (VI) has a carbon chain of structure $-NH-CO-CH_2-N(C_2H_5)_2$. Amphetamine (VII), a simple $-CH_2-CH(CH_3)-NH_2$ substituted benzene, belongs to a major class of central nervous system (CNS) stimulants. Indole alkaloids based on the serotonin structure have a carbon chain substituted in the heterocyclic ring and ending in a nitrogen atom with different degrees of methyl substitution, e.g., 5-HT (VIII). Clenbuterol (IX) is a growth-promoting drug in the beta-agonist class of drugs, with the ability to induce weight gain and a greater proportion of muscle to fat. Clenbuterol and anti-asthmatic drugs such as salbutamol (X) have identical carbon chains bonded to benzene rings with differing substituents as shown in (IX) and (X). Propranolol, a β-adrenoceptor blocker, (XI), has a carbon chain 1-substituted in a naphthalene ring, ending in $-CH(CH_3)_2$ with ether, alcohol and amine groups in the chain.

Fragmentation of these amine-containing side chains can dominate electron impact mass spectrometry (EI-MS) as is the case for phenothiazines and can therefore be of value in structural characterisation of these molecules. The much less investigated ESI-MS^n of such phenothiazines and other drugs containing amine side chains results in fragmentations of these side chains that are of use in structural characterisation. Furthermore, the side chains can be modified/lost in metabolic reactions of the parent molecules as is the case for chlorpromazine (N-oxide formation) and flurazepam (ultimate loss of the side-chain) which is therefore of value in the characterisation of metabolites by ESI-MS^n.

This paper therefore presents a study of the ESI-MS^n of these selected drugs with amine-containing side chains with a view to establishing rules of fragmentation that can be used for the characterisation of such drugs and their metabolites. QTOF-MS/MS is used to support the identity of proposed fragments and comparison is also made with EI-MS data where available. It is intended that the acquisition of this information will also be of value in the characterisation of unknown drugs, isolated from natural products, for example.

In addition, amphetamine, clenbuterol, flurazepam and methadone can be identified and determined in mixtures at low ng/ml concentrations by the application of HPLC–ESI-MS which can also be used for their analysis in saliva samples.

2. Experimental

2.1. Instrumentation and chemicals

MS^n characterisation of the drug compounds was achieved using the Classic LCQ™ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) utilising electrospray ionisation (ESI). 1.0×10^{-5} mol l^{-1} solutions of the drugs in methanol, also containing 1% acetic acid, were infused into the ESI probe at a rate of 10 μl/min. The collision energy was kept at the instrument default value of 25% (arbitrary unit set by the software). The sheath gas flow was set to 50 (arbitrary unit defined by the software) and the auxiliary gas to 5. The capillary temperature was set to 250 °C and the spray voltage to 3.5 kV for the MS^n studies. For MS^n characterisation of the compounds the most intense peak in the mass spectrum, i.e. $[M + H]^+$, was chosen for MS^2 analysis, providing first generation product ions in this mode. The process was repeated for MS^3 and MS^4. The most intense peak from the previous analysis was chosen for further characterisation at each MS^n stage. Only signals of at least 5% abundance that are entirely consistent and reproducible are reported here. An isolation width of 1u was used for the various MS^n stages. Nitrogen gas for the Classic LCQ™ was delivered from a Whatman nitrogen generator (Whatman Inc., Haverhill, MA, USA), while the helium damping gas (99.999% purity) present in the ion-trap was obtained from BOC Medical Gases (Guildford, Surrey, UK).

QTOF-MS was carried out with a Micromass Q-Tof Ultima API mass spectrometer. 10^{-6} mol l^{-1} solutions of the drugs in methanol also containing 1% formic acid were directly infused into the ESI probe at 2 μl min^{-1}. The spray voltage was set to 2 kV with the source temperature at 80 °C and the desolvation temperature at 150 °C. Nitrogen was delivered from a Peak Scientific Nitrogen Generator set at 100 psi and resulted in a nebulising gas flow of 50 l h^{-1} and a desolvating gas flow of 300 l h^{-1}. Argon (99.999% purity) was used as the collision gas with 10 eV energy used in the MS mode and 20–40 eV energy used in the MS/MS mode. The spectrometer was calibrated with sodium formate and adenosine, with lock mass of 268.1040, being used as an internal standard for each sample. The resolution of the spectrometer was ca. 10,000 for the $[M + H]^+$ ions of the drug compounds. Accuracy of measured mass in the QTOF-MS/MS experiments was 10 ppm or less.

HPLC–ESI-MS utilised a reverse phase C_{18} column (Luna 5μ C18, 150 mm × 4.6 mm, Phenomenex) and a binary gradient mobile phase comprising MeOH/H_2O/formic acid (80 + 20 + 0.1) as solvent A and MeOH/H_2O/formic acid (20 + 80 + 0.1) as solvent B. Initially the proportions were 100% A, 0% B and then altered in a linear gradient to 0% A, 100% B over a period of 10 min, then to 100% A, 0% B over 1 min and

held at 100% A, 0% B for 9 min at a flow rate of 0.5 ml/min with injection volume of 50 μl.

Saliva extractions were carried out using spiked samples with a concentration of 5×10^{-7}. 1 ml samples were extracted using 1 ml 40% sodium chloride solution and three successive washes with 5 ml dichloromethane. The dichloromethane was evaporated at room temperature and the samples reconstituted to 1ml with mobile phase A.

Methanol, acetic acid, dichloromethane, formic acid and sodium chloride were obtained from BDH (Poole, Dorset, UK). All drug compounds were obtained from the Forensic Science Association of Northern Ireland (Carrickfergus, NI). The stated purity of these compounds was at least 99%. 10^{-3} mol l^{-1}, standard solutions of the drugs were prepared by dissolving an appropriate mass in 25 ml of methanol.

3. Results and discussion

It should be noted that the LCQ does not give high accuracy mass measurements necessary for unequivocal identification of the products of the fragmentation processes so QTOF-MS/MS is used to support the identity of proposed fragment ions. In the LCQ studies only the major signal (100% abundance) has been selected for further characterisation at each MS^n stage. There are additional peaks at lower intensities that can give rise to a fingerprint of the fragmentation pattern, assisting further in the structural characterisation of the drugs in question. These fingerprints are particularly examined in the MS and MS^2 modes when, in the latter case, the $[M + H]^+$ ion is fragmented. Fingerprints in later MS^n modes are generally of lesser value since the ion that is being fragmented may be substantially different than $[M + H]^+$ and have involved ring closure, ring contraction, etc., although losses of marker species such as NO_2, CO, S, etc., from ring systems can be of diagnostic value.

The ESI-MS^n behaviour of the eleven drugs with amine-containing side chains is summarised in Table 1.

3.1. Drugs with a carbon chain ending in a tertiary amine group

3.1.1. Carbon side chain ending $N–CH_2–CHR–CH_2–N(CH_3)_2$

Chlorpromazine (I) and trimipramine (II) lose 45u from the chain end as the neutral secondary amine $HN(CH_3)_2$ in a charge site initiated fragmentation of the relatively weak C–N bond (bond energy 293 kJ mol^{-1}). The loss of such amines is modestly energetically favourable with values of $\Delta H_f^\circ = -28.0$ and -48.5 kJ mol^{-1} for methylamine(g) and ethylamine(g), respectively. The loss of the amine is confirmed by QTOF-MS/MS with *m/z* signals at 274.0506 ($C_{15}H_{13}NSCl$) and 250.1655 ($C_{18}H_{20}N$) for chlorpromazine and trimipramine, respectively. The loss of the amine would result initially in the formation of an unstable primary carbonium ion, $–CHR–CH_2^+$ and finally result in the formation of an alkene, $–CR{=}CH_2$, with the positive charge now residing on the heterocyclic nitrogen atom. This alkene is subsequently lost as 28u for chlorpromazine and 42u for trimipramine yielding signals in the MS mode at *m/z* 246.6 for chlorpromazine and at 208.2 for trimipramine. These fragment signals are observed for both in-source fragmentation, i.e., MS mode and in MS^n modes. Again QTOF-MS/MS supports the loss of alkene with *m/z* signals at 246.0190 ($C_{13}H_9NSCl$) and 208.1137 ($C_{15}H_{14}N$) for (I) and (II), respectively. Surprisingly, when considering the relatively low energy conditions in the ion-trap, a chlorine radical would appear to be lost after loss of the amine with a signal at *m/z* 239.2 in MS^2 for chlorpromazine but again this is borne out in the QTOF-MS/MS studies with a signal at *m/z* 239.0798 corresponding to $C_{15}H_{13}NS$. This 35u loss would characterise an aromatic Cl atom since aliphatic Cl atoms are not fragmented in this manner as an in-house MS^n study of chloramphenicol $\{p\text{-}NO_2C_6H_4–CHOH–CH(NHCOCHCl_2)–CH_2OH\}$ has shown. A further diagnostic signal for the presence of S in chlorpromazine is observed at *m/z* 214.3 using MS^3 and MS^4, which is supported by QTOF-MS/MS with a signal at *m/z* 214.0466 ($C_{13}H_9NCl$). Furthermore, the C–N bond which involves the N atom of the cyclic 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 (*m/z* 86.0949, $C_5H_{12}N$, using QTOF-MS/MS) and the corresponding ion for trimipramine at *m/z* 100.2 (*m/z* 100.1112, $C_6H_{14}N$ using QTOF-MS/MS). The latter ion for trimipramine has also been observed to fragment to $CH_2{=}N^+(CH_3)_2$ at *m/z* 58.1 at MS and MS^3, a signal not observed using QTOF-MS/MS. The MS^n behaviour of chlorpromazine is given in Fig. 2 and the MS^n mass spectra of trimipramine are given in Fig. 3.

Such ESI-MS^n behaviour should be compared to that observed by EI-MS [4]. 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 $CH_3CH{=}N^+(CH_3)_2$. Promethazine's isomer, promazine, with side-chain $N–CH_2–CH_2–CH_2–N(CH_3)_2$ fragments at all of the shown bonds yielding signals at *m/z* 86 and 58 (base peak), observed for chlorpromazine and trimipramine, respectively using ESI-MS^n. These marker signals at relatively low *m/z* values are also observed for the phenothiazines, acepromazine and propionylpromazine, using EI-MS.

3.1.2. Carbon side chain ending $–CR_2–CH_2–CH(CH_3)–N(CH_3)_2$

Methadone, (III), where R = C_6H_5, represents this type of molecule. In common with trimipramine in Section 3.1.1, the amine, $HN(CH_3)_2$, followed by the alkene, $CH_2{=}CHCH_3$, are lost in the MS stage with signals at *m/z* values 265.5 and 223.5, respectively, supported by QTOF-MS/MS as $C_{19}H_{21}O$ and $C_{16}H_{15}O$. The former signal is again observed at MS^2 and the latter at MS^3. At MS^3, the deaminated species gives rise to

Table 1
ESI-MS^n fragmentation data for 11 drug compounds with amine-containing side chains

m/z signals	MS	MS^2	MS^3	MS^4
Chlorpromazine (I)	*319.5* $[M + 1]^+$, 274.545u loss of $NH(CH_3)_2$, 246.6 further loss of $CH_2{=}CH_2$, 86.1 $CH_2{=}CH\text{-}CH_2NH^+(CH_3)_2$	*273.9* 45u loss of $NH(CH_3)_2$, 246.1 further loss of $CH_2{=}CH_2$ from *m/z* 273.9, 239.2 loss of Cl from *m/z* 274.0	*246.1* as in MS^2, 242.0 loss of S from MS^2 base peak, 239.2 loss of Cl from MS^2 base peak, 214.3 S loss from MS^3 base peak	214.3 as in MS^3, 211.2 loss of Cl from MS^3 base peak, 210.2 loss of HCl from MS^3 base peak
Trimpramine (II)	*295.1* $[M + 1]^+$, 250.2 45u loss of $NH(CH_3)_2$, 208.2 further loss of $CH_3CH{=}CH_2$ (42u), 100.2 $CH_2{=}C(CH_3)CH_2NH^+(CH_3)_2$, 58.1 $CH_2{=}N^+(CH_3)_2$	250.1 45u loss of $NH(CH_3)_2$ from $[M + 1]^+$, 208.1 further loss of $CH_3CH{=}CH_2$, *100.1* as in MS	58.1 as in MS	
Methadone (III)	*310.3* $[M + 1]^+$, 265.5 loss of 45u from $[M + 1]^+$, i.e., $HN(CH_3)_2$, 223.5 further loss of 42u, i.e., $CH_2{=}CHCH_3$	*265.5* as in MS	*247.1* loss of 18u from MS^2 base peak/H_2O, 223.5 as in MS, 219.1, 187.0, 159.1, 105.1 unidentified	219.2, 205.2 unidentified
Flurazepam (IV)	*388.1* $[M + 1]^+$, 315.4 73u loss of $HN(C_2H_5)_2$, 282.7 unidentified	*315.1* as in MS	303.0 unidentified, 295.2HF loss from MS^2 base peak, 289.0 unidentified, 287.0 CO loss from MS^2 base peak, *272.0* diazepine ring contraction, 271.1 unidentified, 244.1 further contraction of diazepine ring, 236.3 loss of Cl from *m/z* 271.1	244.0 as in MS^3
Procaine (V)	*237.5* $[M + 1]^+$, 225.5 unidentified, 207.5 unidentified, 181.4 unidentified, 164.5 loss of $HN(C_2H_5)_2$/73u	*164.1* as in MS, 120.1 further loss of CH_3COH, 100.0 $CH_2{=}CH{-}NH^+(C_2H_5)_2$	120.1 as in MS^2	
Lignocaine (VI)	*235.5* $[M + 1]^+$, 86.4 $CH_2{=}N^+(C_2H_5)_2$	*86.4* as in MS	57.9 $CH_2{=}NH^+(C_2H_5)$	
Amphetamine (VII)	*136.2* $[M + 1]^+$, 119.2 NH_3 loss, 91.4 further loss of $CH_2{=}CH_2$, 74.2 unidentified	*118.9* as in MS	91.2 as in MS	
Clenbuterol (IX)	*277.2* $[M + 1]^+$, 259.4 loss of H_2O, 203.4 further loss of $(CH_3)_2C{=}CH_2$	*258.9* as in MS, 203.0 as in MS	*202.9* as in MS and MS^2, 167.1 loss of HCl from MS^3 base peak, 132.1 further loss of Cl	167.0as in MS^3, 132.1 as in MS^3
5-HT (VIII)	*177.0* $[M + 1]^+$, 160.3 loss of NH_3	*160.2* loss of NH_3	*132.2* loss of CO	115.2 further loss of NH_3
Salbutamol (X)	*240.8* $[M + 1]^+$, 222.5 loss of H_2O, 205.5 unidentified, 181.5 unidentified, 166.3 further loss of $(CH_3)_2C{=}CH_2$ from *m/z*, 222.5, 148.4 further loss of H_2O	*222.0* as in MS, 166.0 as in MS	*166.0* as in MS and MS^2, 148.0 further loss of H_2O	148.0 as in MS and MS^3
Propranolol (XI)	*260.6* $[M + 1]^+$	242.1 loss of H_2O, 218.1 loss of $CH_3CH{=}CH_2$ from $[M + 1]^+$, *183.1* loss of $H_2N\text{-}CH(CH_3)_2$ from dehydrated ion, 157.1 loss of C_2H_2 from deaminated and dehydrated ion, 116.1 $CH_3CH(OH)CH_2NH^+{=}C(CH_3)_2$	165.3 unidentified, 155.2 loss of $CH_2{=}CH_2$ from deaminated and dehydrated ion, 141.2 unidentified	

The italic *m/z* values correspond to those ions that are selected for sequential product ion fragmentation experiments (MS^n).

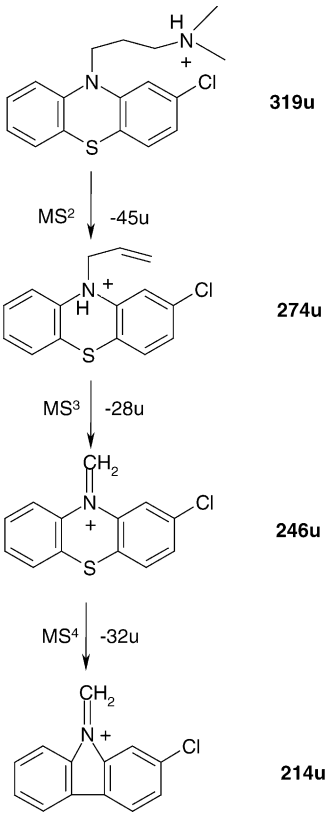

Fig. 2. MS^n fragmentation pathway of chlorpromazine.

a signal at *m*/*z* 247.1 corresponding to a dehydration process ($C_{19}H_{19}$ using QTOF-MS/MS). Other signals are observed at MS^3 and MS^4 stages that are as yet unexplained and are not believed to be diagnostic of particular functional groups in the original molecule.

Ortelli et al. [5] have also observed the $[M + H]^+$ ion at *m*/*z* 310 and fragment ions at *m*/*z* 265 and 223 in their enantioselective analysis of methadone in saliva by HPLC–ESI-MS. The enantiomeric ratio is important to determine since there is individual variability following administration of the racemate with the analgesic potency of the *R*-isomer being 50 times greater than the *S*-isomer.

The EI-MS behaviour is substantially different in that a base peak of *m*/*z* 72 is observed with a variety of small signals including one at 223 [4].

3.1.3. Carbon side chain ending $N–CH_2–CH_2–N(C_2H_5)_2$

The 1,4-benzodiazepine, flurazepam (IV) has the amine-containing side-chain $N–CH_2–CH_2–N(C_2H_5)_2$. On application of ESI-MS^n the tertiary amine entity undergoes charge site initiated fragmentation at the MS stage to give loss of the neutral molecule $HN(C_2H_5)_2$, i.e., a 73u loss resulting in an ion at *m*/*z* 315.4 initially containing the unstable primary carbonium ion, $N–CH_2–CH_2^+$ which rearranges to $N–CH{=}CH_2$. QTOF-MS/MS supports this by observation of a base peak at *m*/*z* 315.0700 corresponding to $C_{17}H_{13}N_2OFCl$. On application of MS^3 the molecule undergoes ring contraction with the diazepine ring losing

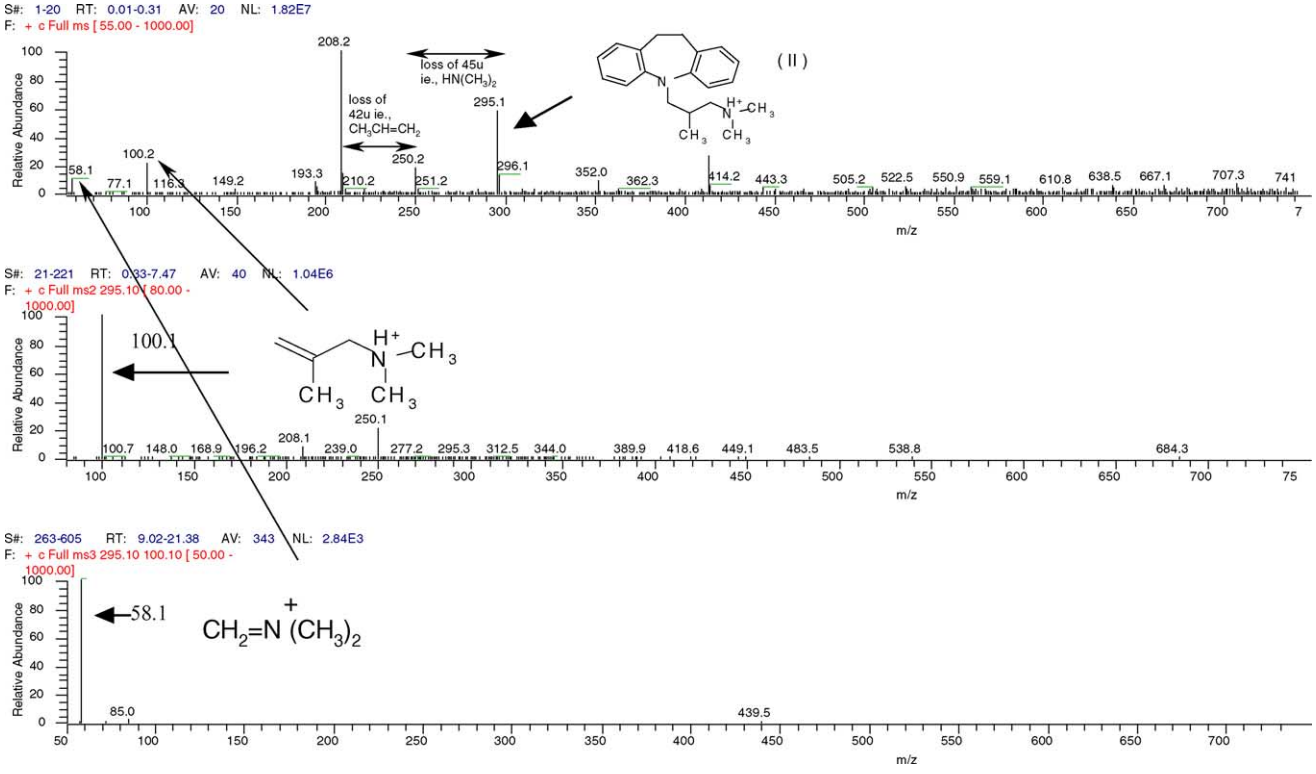

Fig. 3. MS^n spectra of imipramine.

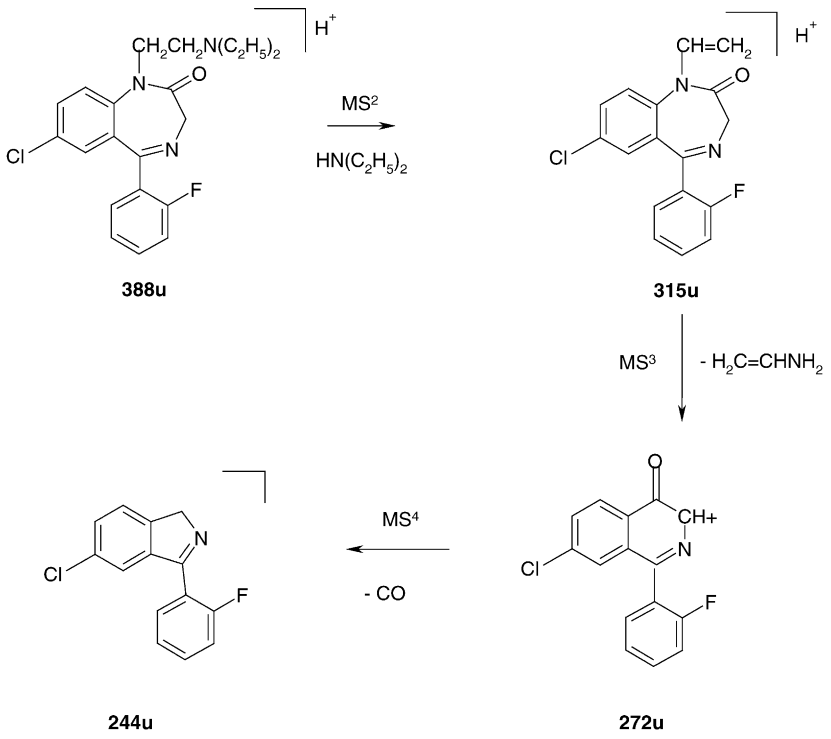

Fig. 4. MS^n fragmentation pathway of flurazepam.

$H_2C{=}CHNH_2$ to form a six membered ring at *m/z* 272.0. A signal at *m/z* 272.0278 ($C_{15}H_8NOFCl$) is observed using QTOF-MS/MS. A lesser signal is observed at *m/z* 287.0, which is believed to be due to loss of ethene/CO. The six membered ring (*m/z* 272.0) undergoes further contraction at MS^4, losing CO to leave a five membered ring, supported by QTOF-MS/MS elemental analysis of $C_{14}H_8NFCl$. Fig. 4 illustrates the MS^n behaviour of flurazepam. It should be noted that flurazepam metabolites *N*-hydroxyethyl-flurazepam and *N*-desmethyl-flurazepam do not possess the amine moiety in the side chain and hence do not give signals in any MS modes corresponding to loss of the tertiary amine which can aid in their structural characterisation [6].

EI-MS of flurazepam is substantially different in that the base peak is observed at *m/z* 86, due to $CH_2{=}N^+(C_2H_5)_2$, with a collection of relatively intense signals in the *m/z* range 99–42 [7]. Lignocaine, discussed in Section 3.1.5, also gives this *m/z* 86 signal on application of ESI-MS^n and EI-MS, due to the same charged species.

3.1.4. Carbon side chain ending –COO–CH_2–CH_2–$N(C_2H_5)_2$

The anaesthetic, procaine, (V), has a carbon chain ending with a tertiary amine function as with flurazepam and this also undergoes charge site initiated fragmentation of the CH_2–N bond with the loss of 73u, i.e., $HN(C_2H_5)_2$ at both MS and MS^2 stages. Brotherton and Yost [8] have screened for drugs in racing animals (e.g., procaine) that contain a terminal $–N(C_2H_5)_2$ group using triple quadrupole mass spectrometry in the neutral-loss scan mode for diethylamine loss. The loss of 73u reduces the chain initially to an unstable carbonium ion, $–COOCH_2–CH_2^+$ and results in the formation of an alkene $–COOCH{=}CH_2$. This, at MS^2 and MS^3, gives rise to further loss of the neutral molecule CH_3COH with a resultant signal at *m/z* 120.1. A further signal at *m/z* 100.0 is observed using MS^2 which corresponds to $CH_2{=}CH–NH^+(C_2H_5)_2$ following fission of a C–O bond. The application of QTOF-MS/MS to procaine gave *m/z* signals at 164.0868, 120.0545 and 100.1201 corresponding to elemental analyses of $C_9H_{10}NO_2$, C_7H_6NO and $C_6H_{14}N$, respectively, which supports the fragmentation pattern proposed (Fig. 5) following the LCQ studies.

The EI-MS of procaine gives relatively intense signals at *m/z* 120, 99 and 86 (base peak) which, as with the EI-MS of flurazepam, shows that the amine-containing side chain is fragmented for procaine to give charged *N*-containing species such as $CH_2{=}N^+(C_2H_5)_2$ at *m/z* 86 and not neutral amines as with ESI-MS^n.

3.1.5. Carbon side chain ending –CO–CH_2–$N(C_2H_5)_2$

Lignocaine/lidocaine (VI) has the chain $–NH–CO–CH_2–N(C_2H_5)_2$ attached to a xylene ring and its ESI behaviour is somewhat similar to its EI behaviour. For ESI it would appear that at least two methylene groups are required adjacent to the end N atom for fission of the C–N bond and loss of $HN(C_2H_5)_2$. Instead in-source fragmentation and MS^2 give rise to a signal at *m/z* 86.4 corresponding to $CH_2{=}N^+(C_2H_5)_2$. Application of MS^3 results in a signal at *m/z* 58.1 corresponding to $CH_2{=}N^+H(C_2H_5)$. It should

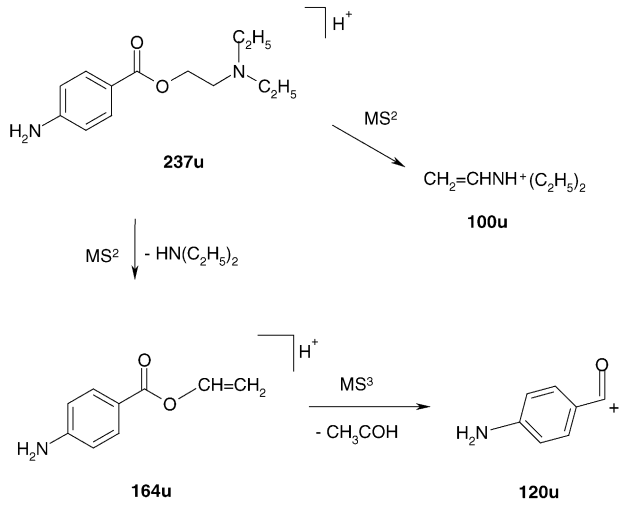

Fig. 5. MS^n fragmentation pathway of procaine.

be noted that signals at *m/z* 86.1 and 58.1 were observed for chlorpromazine and trimipramine, respectively, using ESI but corresponded to different quaternary nitrogen ions. EI of lignocaine/lidocaine also gives signals at *m/z* 86 and 58 [9]. 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 stabilised by virtue of resonance with the xylene ring.

The application of QTOF-MSMS to lignocaine gave an *m/z* signal at 86.0976, which corresponds to elemental analysis of $C_5H_{12}N$, which confirms the fragmentation pattern proposed following the LCQ studies.

Dirkikolu et al. [10] have recently used direct infusion ESI-MS/MS to detect metabolites 3-hydroxylidocaine and its glucuronide in equine urine. The diagnostic product ion in both cases was that at *m/z* 86 as is expected from the fact that these metabolic products involve modification of the substituted benzene ring leaving the amine-containing side chain unaltered.

3.1.6. Indole alkaloids with carbon side chain ending in $-CH_2-CH_2-NR_2$

Indole alkaloids based on the serotonin structure have a carbon side chain substituted in the heterocyclic ring and ending in a nitrogen atom with different degrees of methyl substitution. All of these compounds that have been subjected to ESI-MS^n, i.e., 5-HT, (VIII), *N′*-methyl 5-HT, bufotenine, and 5-HTQ, give rise to charge site initiated fragmentation of the appropriate C–N bond with H atom transfer to lose, respectively, NH_3, CH_3NH_2, $(CH_3)_2NH$ and $(CH_3)_3N$ [11]. Protonation of the aliphatic N atom (pK_a 10–11 in aqueous solution at 20–25 °C) is considerably more likely than of the indole nitrogen (pK_a of indole is −2.3 under similar conditions). The resulting positive ion in all cases at *m/z* 160 is unlikely to lose $CH_2{=}CH_2$ due to conjugation with the ring system. Instead it has been proposed [11] that CO and NH_3 are lost at MS^3 and MS^4 stages. Fig. 6 illustrates the MS^n behaviour of these indole alkaloids. QTOF-MS/MS supports this with *m/z* signals at 132.0775 ($C_9H_{10}N$) and 115.0508 (C_9H_7), respectively. The EI of 5-HT gives signals among others corresponding to fission of the three bonds in the amine-containing side chain, i.e., two C–C bonds and one C–N bond [12].

3.2. Drugs with a carbon side chain ending with a primary amine group

Amphetamine, $C_6H_5{-}CH_2{-}CH(CH_3)NH_2$, (VII), shows an $[M + H]^+$ ion at *m/z* 136.2 with minor signals at *m/z* 119.2, 91.4 and 74.2. The *m/z* 119.2 signal corresponds to NH_3 loss ($\Delta H_f^\circ = -46.1\,kJ\,mol^{-1}$ for the gas) by charge site initiated fragmentation. In keeping with this observation, QTOF-MS/MS assigns an elemental formula to an *m/z* 119.0302 signal of C_9H_{11}. Unlike the case with drugs such as trimipramine, $CH_3CH{=}CH_2$ is not lost from the deaminated ion of amphetamine although the signal at *m/z* 91.4 could correspond to loss of ethene (28u). QTOF-MS/MS of the signal at *m/z* 91.0874 gave elemental analysis of $C_7H_7^+$, the tropylium ion. MS^2 and MS^3 give single signals at *m/z* 118.9 and 91.2, respectively. The EI-MS of amphetamine is somewhat different giving a base peak at *m/z* 44 and a small signal at 91 [4].

3.3. Drugs with a carbon side chain that includes a secondary amine group

3.3.1. Chain ending in $-CH(OH)-CH_2-NHC(CH_3)_3$

Clenbuterol (IX) undergoes electrospray ionisation 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. Two additional signals are observed in this MS mode at *m/z* values 259.4 and 203.4 corresponding to H_2O loss and further loss of $(CH_3)_2CH{=}CH_2$ to give a modified chain $-CH{=}CH{-}NH_3^+$ which is resonance stabilised by conjugation to the substituted benzene ring. The $[M + H]^+$ species loses H_2O at the MS^2 stage to give a signal at *m/z* 258.9 followed by the loss of 56u 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 yields 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, also observed at MS^4, appears to correspond to removal of HCl with formation of a fused nitrogen-containing ring from the amine-containing side chain. The loss of HCl is energetically favourable in that for the gaseous state $\Delta H_f^\circ = -92.3\,kJ\,mol^{-1}$. It should be noted that the aromatic amine functional group is again stable in the MS^n study as was the case with procaine (V). The MS^n behaviour of clenbuterol is illustrated in Fig. 7.

The application of QTOF-MSMS to clenbuterol gave *m/z* signals at 259.0768, 203.0133, 167.0348 and 132.0638 corresponding 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, which

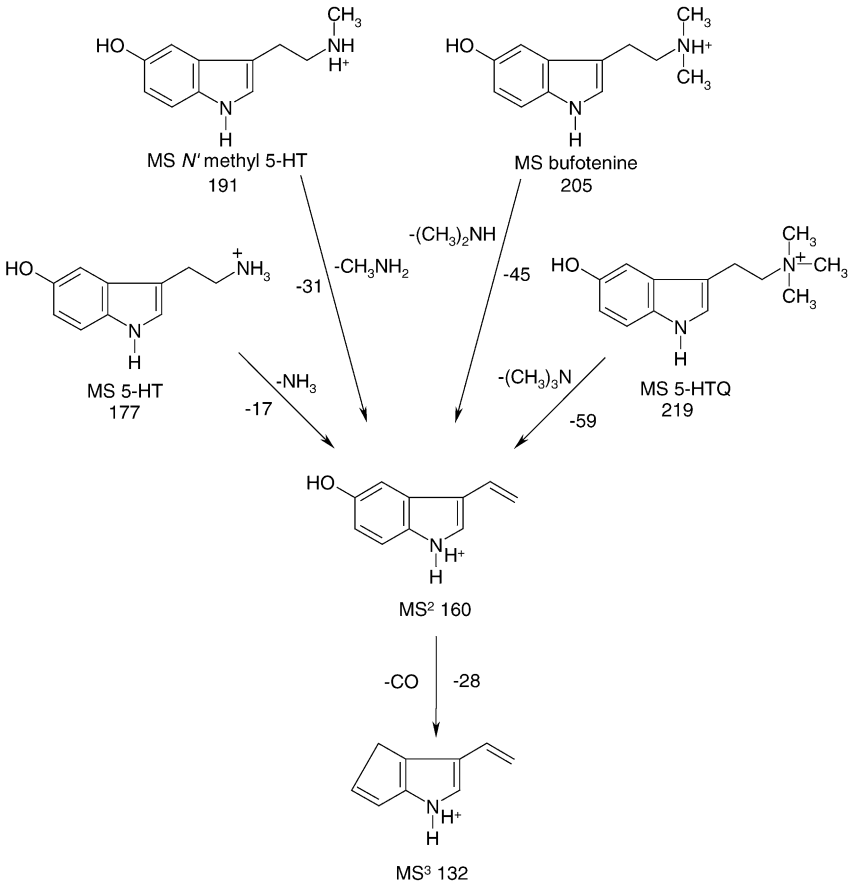

Fig. 6. MS^n fragmentation pathway of indole alkaloids.

supports the fragmentation pattern proposed following the LCQ studies.

Sauer et al. [13] have used ESI-MS/MS to study the biotransformation of clenbuterol by bovine liver and found a glucuronide at *m/z* 453 and a hydroxylated metabolite at *m/z* 293. This latter metabolite fragments to give signals at *m/z* 275 (loss of H_2O) and *m/z* 219 [loss of $(CH_3)_2C{=}CH_2$], mass losses that are paralleled in the parent molecule suggesting aliphatic or aromatic *N*-hydroxylation. It is therefore suggested that these two metabolic routes could be differentiated by application of ESI-MS^n, as presented in this paper for clenbuterol, since the MS^4 stage is believed to involve cyclisation of the amine-containing side chain to the substituted benzene ring with loss of HCl. This cyclisation will probably be affected by having an hydroxylated amine-containing side chain.

The related β-agonist, salbutamol (X), with different substituents in the benzene ring, gives rise to signals at *m/z* 240.8, 222.5, 166.3 and 148.4 when fragmentation is achieved in-source using ESI-MS^n. This again corresponds to $[M + H]^+$, loss of H_2O, loss of the *t*-butyl group as 2-methylpropene and further loss of H_2O. These signals are also observed at MS^2 and MS^3. The MS^3 signal at *m/z* 148.0 corresponds to H_2O loss from the second aliphatic OH group. Selection of the 166.0 signal at MS^4 gives rise to a single signal at 148.0. Fig. 8 shows the ESI-MS^n fragmentation pathway for salbutamol using predominant fragment ions.

The application of QTOF-MSMS 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 supports the fragmentation pattern proposed following the LCQ studies. Sauer et al. [13] have used ESI-MS/MS to identify the *O*-phenylglucuronide of salbutamol at *m/z* 416 in the study of its biotransformation by bovine liver.

When subjected to EI-MS clenbuterol gave a variety of signals at *m/z* 243 (loss of H_2O + CH_3), 190 {loss of 86u, i.e., $(CH_3)_3C{-}NH{-}CH_2{}^+$}, 127 (origin unknown), 86 {$(CH_3)_3C{-}NH{-}CH_2{}^+$}, 70 (origin unknown), 57 {$(CH_3)_3C^+$}, 41 (origin unknown) and 30 ($CH_2NH_2{}^+$). As expected PCI/CH_4 gave signals at higher *m/z* values including the $[M + H]^+$ signal at *m/z* 277 with only the *m/z* signal at 86 at *m/z* < 100 [4].

3.3.2. Carbon side chain ending in $-O-CH_2-CH(OH)-CH_2-NH-CH(CH_3)_2$

Propranolol, (XI), with a structure that involves the above chain being a substituent in naphthalene through the oxygen atom in the 1-position, gives an $[M + H]^+$ ion at *m/z* 260.6 in the MS mode and no other signals of intensity greater

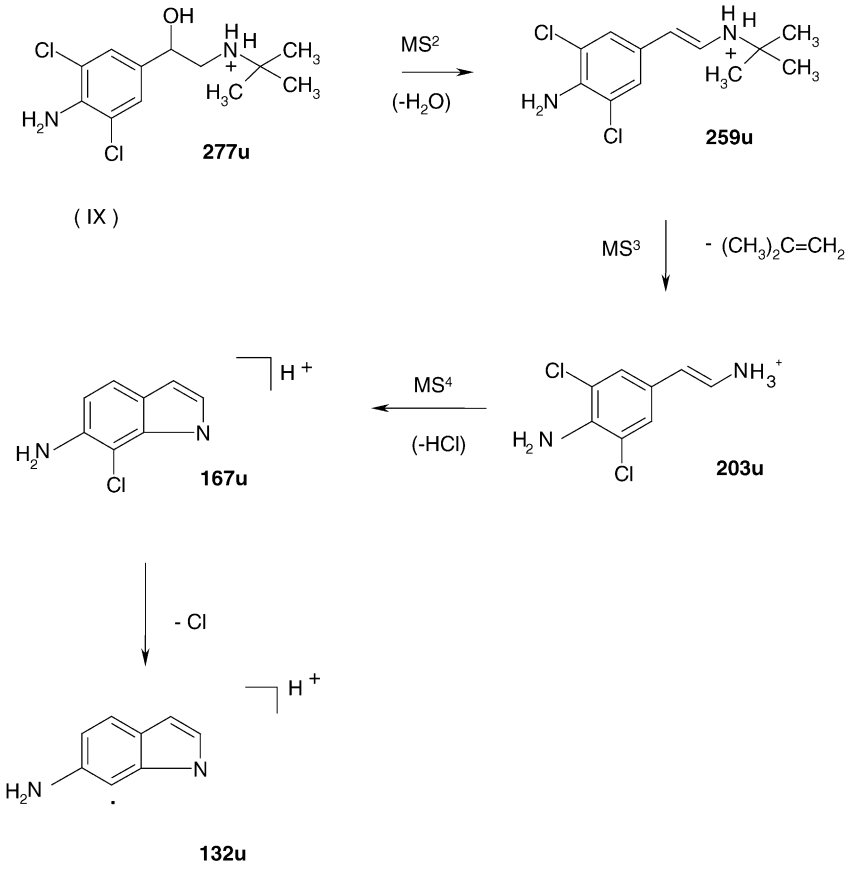

Fig. 7. MS^n fragmentation pathway of clenbuterol.

than 5% relative abundance. At MS^2 signals are observed at 242.1 (loss of H_2O), 218.1 (loss of $CH_3CH{=}CH_2$ from $[M + 1]^+$), 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 deaminated and dehydrated species) and a signal at *m*/*z* 116.1 which suggests C–O bond fission to give the ion $CH_3CH(OH)CH_2NH^+{=}C(CH_3)_2$. Beaudry et al. [14] have used this latter signal to identify metabolites of propranolol in which the amine-containing side chain is not modified (e.g. hydroxylated ring and glucuronide metabolites) by

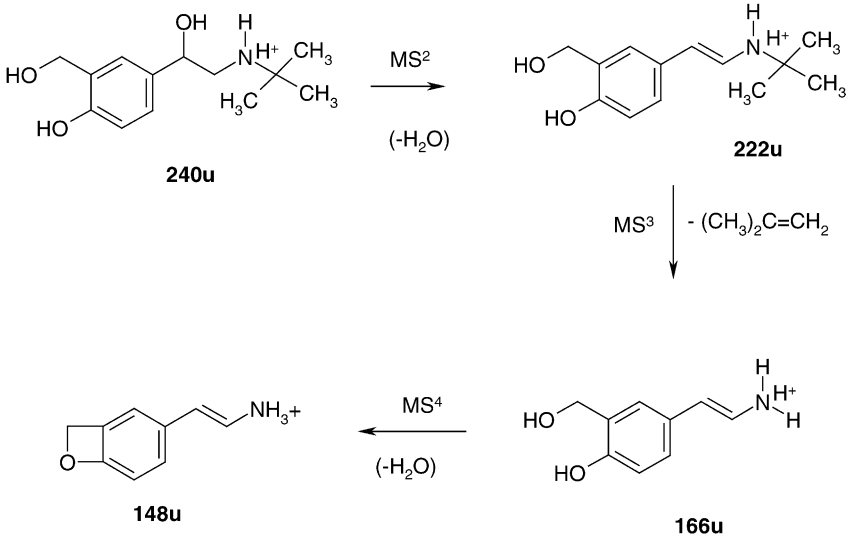

Fig. 8. MS^n fragmentation pathway of salbutamol.

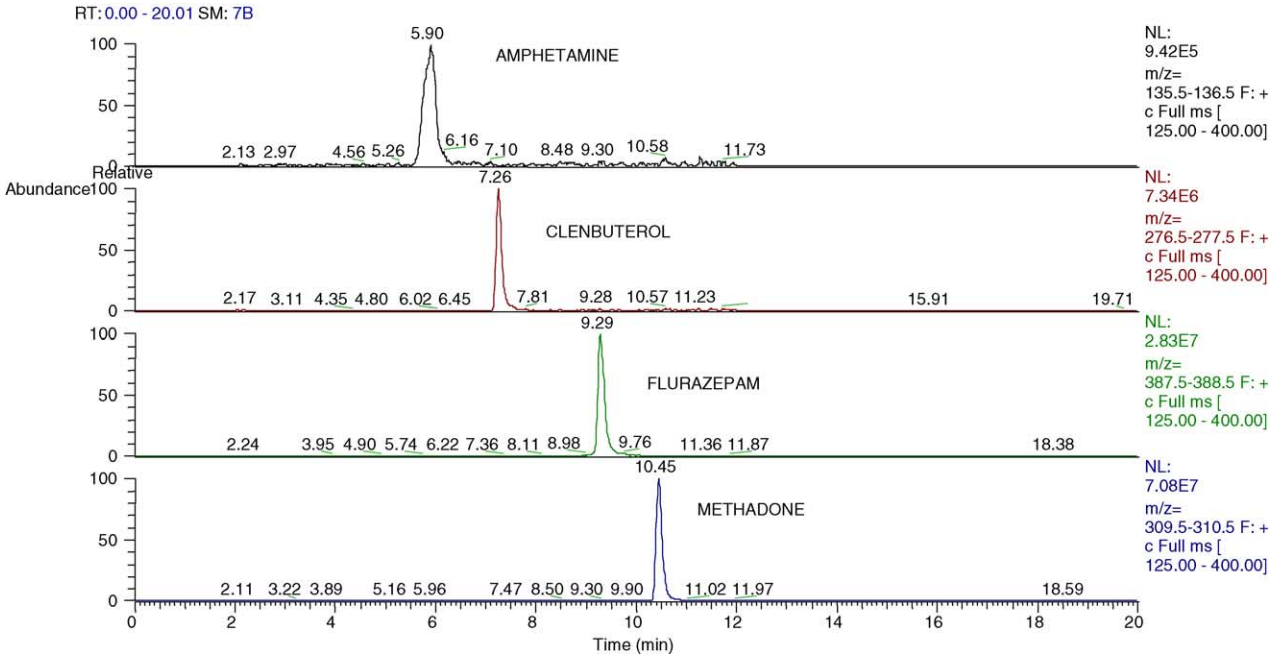

Fig. 9. HPLC–ESI-MS of a mixture of 5.10^{-7} mol l^{-1} concentrations of amphetamine, clenbuterol, flurazepam and methadone.

performing a precursor ion scan of *m/z* 116. The identification of glucuronide metabolites was made by monitoring the characteristic loss of *m/z* 176 (sugar base) in constant neutral loss scan mode. To confirm the presence of hydroxylated metabolites, a specific constant neutral loss scan for *m/z* 144 + 16*n* (where *n* = 0, 1 and 2) was carried out. Finally, in this study, MS^3 using the 183.1 signal gives a base peak at 155.2, which amounts to an ethene loss, not observed with clenbuterol and salbutamol since ethene's double bond is not conjugated to the naphthalene ring. Two unidentified signals are observed at *m/z* 165.3 and 141.2.

The application of QTOF-MSMS to propranolol gave *m/z* signals at 218.1158, 183.0776, 157.0604, 155.0803 and 116.1016 corresponding 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 supports the fragmentation pattern proposed following the LCQ studies.

3.4. HPLC–ESI-MS study of amphetamine, clenbuterol, flurazepam and methadone

Each of the drug compounds was chromatographed at an initial concentration of 5×10^{-7} mol^{-1} using ESI-MS detection followed by serial dilution. R^2 values for the calibration plots were 0.9948 or better with coefficients of variation of between 5.25% and 9.66% at 5×10^{-7} mol^{-1}. HPLC–ESI-MS of a mixture of the four drugs is given in Fig. 9 showing complete baseline resolution and the ability of this technique to identify and quantify the particular drug in question.

3.5. Trace analysis of drugs in saliva

One millilitre saliva samples were spiked with the drugs mixture to give a concentration of 5×10^{-7} mol^{-1} for amphetamine, clenbuterol, flurazepam and methadone and were solvent extracted using 1 ml 40% sodium chloride solution and 3×5 ml dichloromethane per sample. Following solvent extraction, the residues were then reconstituted to the original volume of 1 ml with mobile phase A prior to the application of HPLC–ESI-MS using selected ion monitoring of the protonated molecular ions at the *m/z* values 136, 277, 388 and 310, respectively. Recoveries were calculated and averaged to give 109.16% with a coefficient of variation of 7.05% for amphetamine, 54.63% with a coefficient of variation of 2.59% for clenbuterol, 73.22% with a coefficient of variation of 5.99% for flurazepam and 79.60% with a coefficient of variation of 2.34% for methadone. The retention times for the HPLC method were 5.90, 7.26, 9.30 and 10.45 min, respectively.

4. Conclusions

From this study certain rules can be formulated with respect to the ESI-MS and subsequent MS^n behaviour of drugs with amine-containing side chains.

1 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 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 can 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, trimipramine and methadone.

Molecules ending with a primary nitrogen atom in its side chain, e.g., amphetamine, lose the amine as ammonia.

2 If there is only one methylene group adjoining the end nitrogen atom as is the case with lignocaine then the end of this carbon chain becomes the detectable charged species as in $CH_2{=}N^+(C_2H_5)_2$ and no neutral amine or alkene is formed.

3 Molecules such as clenbuterol and salbutamol which have a secondary amine-containing carbon chain $-CH(OH)-CH_2-NH-C(CH_3)_3$ lose H_2O followed by the end substituted carbon group as alkenes $(CH_3)_2C{=}CH_2$ on application of MS and MS^n.

Propranolol with the chain $-CH(OH)-CH_2-NH-CH(CH_3)_2$ loses H_2O, $CH_3CH{=}CH_2$, $NH_2-CH(CH_3)_2$ and C_2H_2 at MS^2 with ethene loss at MS^3 from the dehydrated and deaminated species.

These MS^n experiments, supported by QTOF-MS/MS data, therefore show certain characteristic fragmentations with respect to the amine-containing side chains. The data provides useful information on the structure of these compounds with amine-containing side chains and can be used in the characterisation of such drugs and their structurally related metabolites. The ESI-MS^n data of such compounds can be held in a database and neutral mass losses/low molecular mass ions cross-referenced with similar data obtained from unknown analytes which should then be of value in their structural characterisation with respect to amine-containing side chains.

The observed neutral mass losses and their structural inferences for these and other molecules are given in Table 1 of a recent publication [1]. This Table contains data from recent publications for low molecular mass molecules collected by the research group at the University of Ulster [1–3,15–18] using the same LCQ and identical experimental conditions.

Finally, amphetamine, clenbuterol, flurazepam and methadone can be identified and determined in mixtures at low ng/ml concentrations by the application of HPLC–ESI-MS which can also be used for their analysis in saliva samples.

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