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Mass Spectrometric Study of a Specific Derivatization Reaction Between *N*,*N*-Dimethylformamide Dimethylacetal and the Ethanolamine Moiety of β-Agonistic Drugs

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In a study designed to examine the nature of a specific reaction which was shown to occur between the ethanolamine moiety of β-agonists and *N*,*N*-dimethylformamide dimethylacetal (DMF-DMA), several mass spectrometric techniques were used to identify the main reaction intermediates and/or products. In particular, the use of fast atom bombardment (FAB) ionization made the study of polar and thermosensitive low molecular mass compounds possible. High-resolution (R ≈ 10 000) and linked-scan experiments performed on reverse-geometry double-focusing mass spectrometers were helpful to confirm structural hypotheses. From this study, it appeared that the secondary amine of the ethanolamine group of β-agonists can react with DMF-DMA (DMA being the active part of the reagent) to give mainly an ethanolamide intermediate. Apart from this ethanolamide intermediate, three reaction products which are partly due to a dehydration step of the ethanolamide that occurs at high temperatures were identified. Two of them are the results of a cleavage of the side-chain which gives a styrene and an isocyanate derivative; the third one corresponds to an azetidine derivative stemming from a side-chain cyclization. These unexpected findings may be of great help for the survey of β-agonist residues. Actually, the above-mentioned reaction products could be easily detected and identified in biological samples by means of gas chromatography (Ross injector, 280 °C) coupled to mass spectrometry (GC/MS); the side-chain cleavage observed during the formation of the styrene and isocyanate derivatives was useful to broaden the range of detection and to facilitate the identification of new analogues. On the other hand, the analysis of ethanolamide intermediates via liquid or thin-layer chromatography coupled to FABMS would also appear to offer valuable analytical solutions.

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INTRODUCTION

β-agonists, so called β-sympathomimetics, are characterized by their structural and pharmacological properties, which are very close to those of catecholamines. These drugs are used as bronchodilators, tocolytics or heart tonics in human and veterinary medicine. During the past 10 years, several studies focused on the effects of such synthetic molecules on growth rate and performances, when administered *per os*, mixed with feedingstuffs. From 1984, it was pointed out that a specific β-agonist, so-called clenbuterol, was able to increase notably the protein to fat ratio[1–4] in athletes and meat-producing animals. In the following years, these properties were also emphasized for other β-agonists such as cimaterol, ractopamine and fenoterol.

Because of their ability to shift nutrients towards protein instead of lipid anabolism, such molecules were gathered under the generic name of 'repartitioning agents.'[5] Basic knowledge on β-agonists concerns mainly the previously mentioned substances.[6] Although β-agonistic drugs have never been licenced for growth-promoting purposes, their use as growth promoters appeared to spread to a large extent, especially in livestock. Since toxicological data on such pharmacological manipulations are lacking, their addition to animal feed has been forbidden within the European Union Member States, notably in France.[7] Further, the illegal use of β-agonists was revealed to be of major concern for public health and some food poisoning cases were reported.[8,9] Faced with such problems, analytical techniques designed for the unambiguous detection and identification of β-agonists in biological matrices had to be developed. Among the available techniques [e.g. thin-layer chromatography (TLC), radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), infrared (IR) spectrometry], mass spectrometry (MS) appeared to be the most efficient tool to provide results which are sensitive and specific enough to allow enforcement action.

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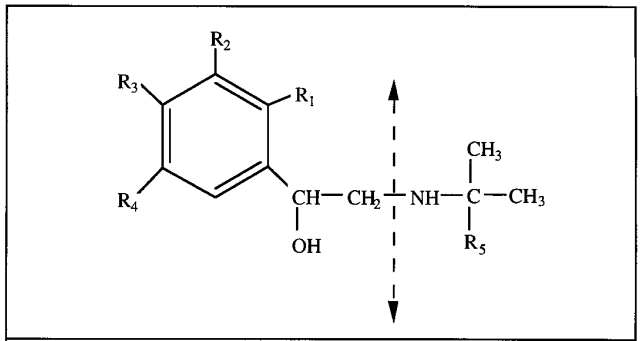
Consequently, several methods based on MS instrumentation have been set up in recent years, among which gas chromatography (GC)/MS or liquid chromatography (LC)/MS techniques [occasionally reinforced by tandem MS (MS/MS) or high-resolution (HR) configurations] seem to be the most powerful. Concerning GC/MS techniques, preliminary trimethylsilylation[10–19] or *tert*-butyldimethylsilylation[20] are commonly used derivatization methods. However, when analysed under electron impact (EI) ionization conditions, these derivatives are well known to give very poor informative data (especially for clenbuterol-like compounds) and their use usually does not permit the identification of residues (according to the required quality criteria[21]) below the 1 ng ml^{-1} level. Consequently, other analytical tools based on different derivatives and/or ionization modes have to be set up, notably for confirmatory purposes. Cyclic boronic derivatives[22–24] were shown to give far more informative data when analysed by means of GC/EIMS. In addition, 2-dimethylsilamorpholine, so-called cyclic dimethylsilylmethylene derivatives,[19,25] also proved to be of major interest for the confirmation step. Unfortunately, these cyclic derivatizations are useful for clenbuterol-like compounds but not easily feasible for β-agonists containing additional hydroxy substituent(s). Therefore, silylated (or cyclic)[14–16,19] derivatives may be analysed as well in the positive chemical ionization (PCI) mode. Further, some workers also emphasized the interest of using acylated derivatives analysed under negative chemical ionization (NCI) conditions[26–28] and MS/MS in the selected reaction monitoring mode (SRM-MS/MS)[17] or high-resolution selected ion monitoring (HRSIM)[24,29] experiments were shown to improve greatly the sensitivity and specificity of analysis. LC/MS techniques are also promising and some methods involving thermospray (TSP),[30] electrospray (ESI)[31] [sometimes combined with isotachophoresis/capillary zone electrophoresis (ITP/CZE)[32]] or atmospheric pressure chemical ionization (APCI)[33] interfaces were recently developed in that field of research.

Apart from the analytical problems due to the poor informative data obtained when monosilylated (hydroxy group of the ethanolamine side-chain of clenbuterol-like compounds) are analysed by GC/EIMS, 'new' illegally used β-agonists may easily elude the investigations usually set up during the screening step, since the corresponding pure substance is not available in control laboratories. Therefore, new analogues are rarely brought to light, especially when mass fragmentographic methods are used for detection.

In this context, the initial aim of the present study was to attempt to make the primary amine of the aromatic ring of β-agonists such as clenbuterol, clenpenterol, clenproperol, brombuterol, NA 1141, mabuterol, mapenterol, cimaterol, cimbuterol (and many others; Fig. 1) react with DMF-DMA to give an N-(N′,N′-dimethylaminomethylene) (N-DMAM) derivative as described in the literature for amino acids[34,35] (Fig. 2) or primary sulfonamides,[36] while a silylation step of the side-chain hydroxy group(s) was maintained. However, in spite of preliminary studies based on multiple combinations of solvents, reagents and reaction conditions,



$R_3 = NH_2$ **$R_1 = H$**	$R_2 = Cl$	$R_4 = Cl$	$R_5 = CH_3$	clenbuterol
			$R_5 = H$	clenproperol
			$R_5 = CH_2OH$	NA1141
			$R_5 = C_2H_5$	clenpenterol
	$R_2 = Br$	$R_4 = Br$	$R_5 = CH_3$	brombuterol
	$R_2 = Cl$	$R_4 = CF_3$	$R_5 = CH_3$	mabuterol
			$R_5 = C_2H_5$	mapenterol
	$R_2 = CN$	$R_4 = H$	$R_5 = CH_3$	cimbuterol
			$R_5 = H$	cimaterol
	$R_2 = Cl$ = Br = H = I = CN = H = Br = Br = Cl = H = Cl = H = H	$R_4 = F$ = F = F = F = F = CF_3 = CF_3 = CN = CN = Br = CH_3 = CH_3 = CH_2OH	$R_5 = ?$	other possible aromatic ring substitutions ...
$R_3 = H$ **$R_1 = H$**	$R_2 = OH$	$R_4 = OH$	$R_5 = CH_3$	terbutaline
			$R_5 = H$	metaproterenol
$R_3 = OH$ **$R_1 = H$**	$R_2 = H$	$R_4 = CH_2OH$	$R_5 = CH_3$	salbutamol
$R_3 = H$ **$R_1 = Cl$**	$R_2 = H$	$R_4 = H$	$R_5 = CH_3$	tulobuterol

Figure 1. Structure of potentially used β-agonists. Note, the site of rupture of the ethanolamine side-chain which is induced by DMF-DMA.

the expected derivatives could not be observed when using GC/MS for separation and detection.[29] On the other hand, a component was surprisingly identified to be a styrene derivative stemming from the reaction. The GC/EI mass spectrum corresponding to the single

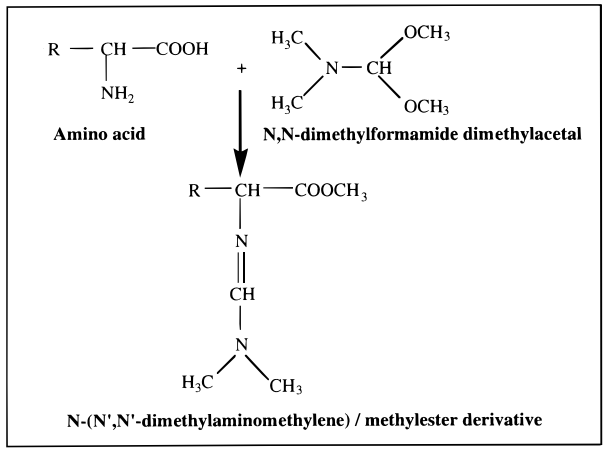


Figure 2. DMF-DMA-derivatized amino-acid to form an N-DMAM/methyl ester derivative.

(a)

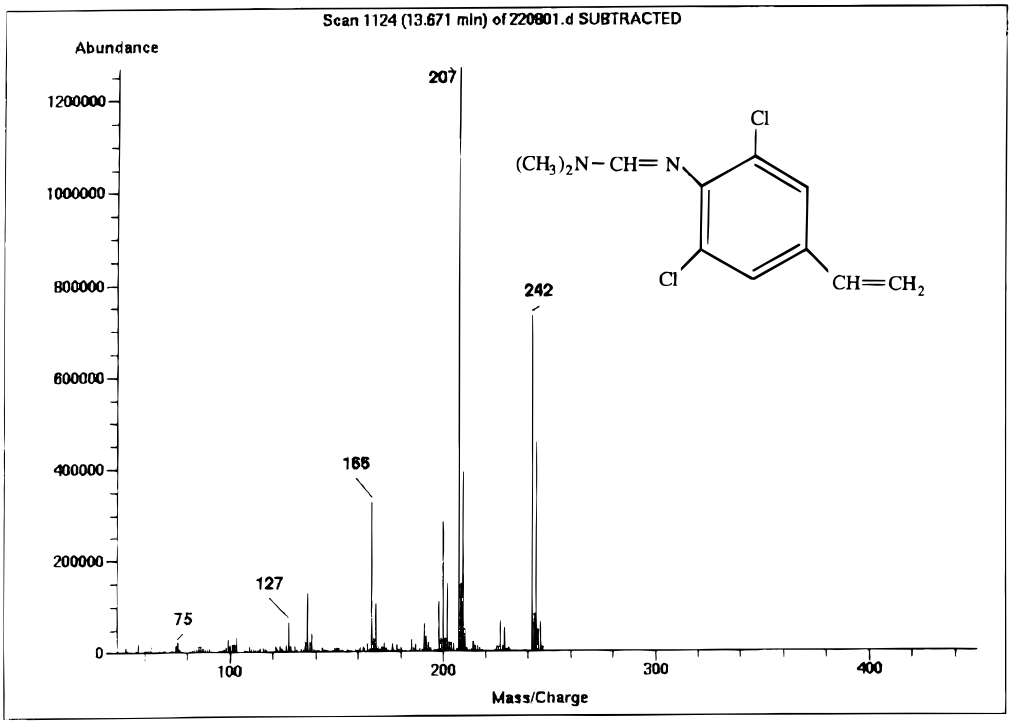


Figure 3. (a) EI mass spectrum of the N-DMAM-styrene stemming from clenbuterol or β-agonists which contain the same aromatic ring moiety as clenpenterol, clenproperol or NA1141 (GC/MS).

styrene obtained in similar conditions for clenbuterol, clenpenterol, clenproperol or NA 1141 (i.e. compounds which are made up of the same aromatic moiety) is given in Fig. 3. For this compound, a molecular ion set at m/z 242.0378, which corresponds to a $C_{11}H_{12}N_2Cl_2$ molecular formula hypothesis, was identified and confirmed by means of PCI and HR ($R \approx 10\,000$) experiments. This result made us believe that the primary amine of the aromatic moiety should react with DMF-DMA to give the expected N-DMAM derivative, while the reaction should affect the secondary amine of the ethanolamine moiety. Therefore, the reaction product was assumed to correspond to the simultaneous loss of the *tert*-butylamine and hydroxy groups of the side-chain. Nevertheless, the potential reactivity of DMF-DMA against secondary amines was difficult to evaluate, as no published data were available. This preliminary result was of prime interest in the previously described analytical field, β-agonists which are illegally used for growth-promoting purposes often resulting from modifications of the aromatic ring substituents (Fig. 1). Moreover, the ethanolamine-containing side-chain may also be modified to a fairly large extent, when compared with well known patented molecules. It has been reported that about 20 different acetophenones can be used for the synthesis of this kind of compound and numerous amines (~ 50) are commercially available for this purpose.[37] One could therefore easily understand the interest in this unexpected effect of DMF-DMA on β-agonists. In addition, the rupture of the side-chain, leading to the formation of a substituted styrene, could also be observed for β-agonists (such as fenoterol, terbutaline or metaproterenol) which do not contain a 4-aminophenyl moiety, but whose aromatic ring is substituted with hydroxy groups (Fig. 3). In that case, DMF-DMA had to be combined with a *tert*-butyldimethylsilylating agent prior to GC/MS analysis.

These results were sufficiently encouraging to lead us to think that this reaction (once sufficiently well understood) should offer valuable analytical solutions, i.e. possibilities to broaden the range of detection (limited until then to the ~ 15 β-agonists available in control laboratories), to simplify mass fragmentographic methods (which is particularly useful for HRSIM investigations) and to facilitate new analogue identification.

Consequently, further experiments based on MS instrumentation had to be carried out in order to identify intermediates and/or other reaction products and to elucidate how DMF-DMA could react with the secondary amine of the ethanolamine moiety of β-agonists.

EXPERIMENTAL

Chemicals

All reagents were of analytical grade. N,N-Dimethylformamide dimethylacetal (DMF-DMA; concentrated Methyl 8) and pyridine (GC quality) were obtained from Pierce (Rockford, IL, USA). N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), propane-1,2,3-triol, toluene over molecular sieve (GC quality) were purchased from Fluka (Buchs, Switzerland), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma (St Louis, MO, USA), perfluorokerosene (PFK) and xenon (4.0) from Koyo Science (Saitamaken, Japan), Ultramark from Interchim

(b)

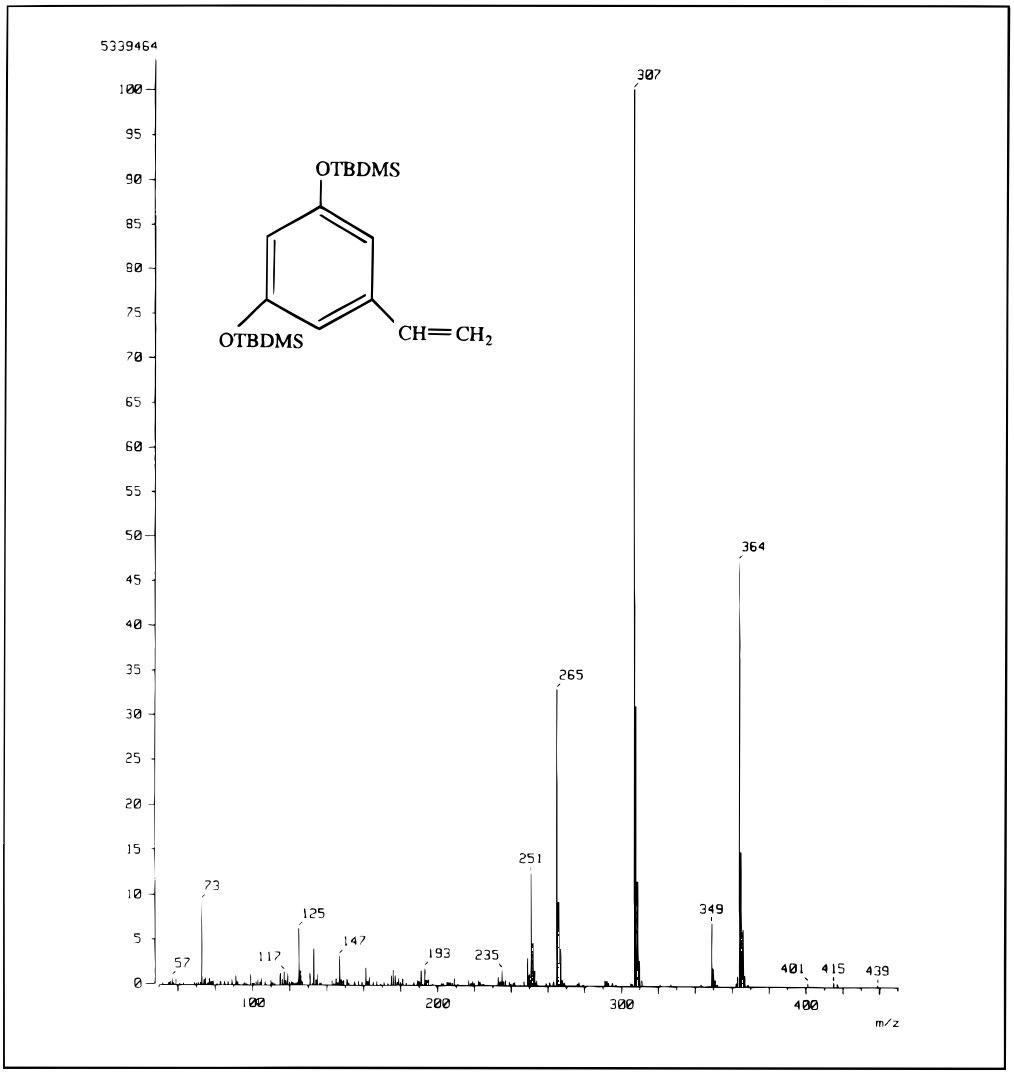


Figure 3. (b) EI mass spectrum of the di-O-TBDMS-styrene stemming from terbutaline or β-agonists which contain the same aromatic ring moiety as fenoterol or metaproterenol (GC/MS).

(Montluçon, France), helium (5.5), methane (4.5) and isobutane (3.5) from Carboxyque (Nantes, France), dicyclohexylcarbodiimide (DCC) from Aldrich (St Quentin Falavier, France) and TLC plates (DC Aufolien, Kieselgel 60W) from Merck (Darmstadt, Germany).

Instrumentation

MS experiments were performed on two reverse-geometry double-focusing SX102 and SX102A mass spectrometers (Jeol, Tokyo, Japan). An SX102A mass spectrometer (EI/CI ion source) was used in the following configurations: GC/MS (GC: Model 5890, Hewlett-Packard (HP, Palo Alto, CA, USA); split/splitless injector), LC/MS (LC: Model 1050, HP) and direct probe (fast atom bombardment (FAB)). For FAB+ investigations, equipment from the 10 Series (Jeol), i.e. FAB ion source, FAB gun, FAB direct probe, FRIT-FAB LC/MS interface, was adapted on this instrument coupled to an Apollo station (HP, Unix system MS-MP7000 (HP and Jeol)). An HPLC system (1050 Series from HP, 100 µl injector) equipped with a Spherisorb column (ODS2, 250 × 4.6 mm i.d., 5 µm, Merck) was used for LC/FRIT-FABMS assay. A linked scan unit was also available and B/E linked-scan analyses were performed on this mass spectrometer. The SX102 mass spectrometer (EI/CI ion source) was used in the following configurations: GC/MS (GC: Model 3300, Varian (Palo Alto, CA, USA); split/splitless or Ross injector), TLC/FABMS, electron impact desorption (DEI) direct introduction. For FAB+ and DEI analyses, equipment from the 10 Series (Jeol), i.e. TLC/MS interface and DEI probe, were adapted on this instrument coupled to a Model 4208 station (Tektronix, Wilsonville, OR, USA; RSX system, JMA DA600 (Jeol and Digital)). A GC/MS quadrupolar apparatus (Models 5890 and 5971, respectively, from HP) equiped with a Model 7673 autosampler and a split/splitless injector was also used in the EI and PCI modes, in order to optimize the experimental conditions.

Analytical conditions

Since the assays which were performed to elucidate the reaction are based on several MS techniques, most of

the analytical conditions required for these specific analyses are appended to the results. However, some technical characteristics mentioned below are common to all corresponding experiments:

GC: OV-1 fused-silica capillary columns (Ohio Valley, Marietta, OH, USA), 30 m × 0.25 mm i.d. with a film thickness of 0.25 μm, oven temperature programmed as follows: 70 °C (2 min), 15 °C min^{-1} up to 200 °C (0 min), 5 °C min^{-1} up to 245 °C (0 min), then 25 °C min^{-1} from 245 to 300 °C (8 min). Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. A Ross injector (Model M33022, SPIRAL, Dijon, France) was used for the identification of the reaction product corresponding to the side-chain moiety, otherwise injections were performed in the splitless mode (1 μl injected, 1 min delay).

Ion source temperature for GC/MS experiments: 250 °C.

Reverse-geometry MS: accelerating voltage (V0): +10 kV.

FAB gun: accelerating voltage of Xe^+ ions: +3 kV.

TLC/MS: 1 scan per 0.2 mm^{-1} (automatic mode).

HR analyses: the results concerning elemental compositions were considered to be acceptable when the mass differences were below 5 u (as an absolute value). Consequently, mass measurement errors will not be systematically mentioned in the text. In addition, the required reaction conditions are also appended to the results.

RESULTS

Study of reaction mechanisms

At the beginning of the present work, fenoterol was considered to be a component of choice for studying the reaction. Because of the structure of this molecule, i.e. a heavy phenol-containing side-chain, we assumed that the second moiety stemming from the rupture of the ethanolamine side-chain would be easily identified when using GC/MS (even with a split/splitless injector). Actually, this last reaction product was presumed to be more difficult to isolate and identify for β-agonists whose secondary amine is substituted with a *tert*-butyl or an isopropyl group.

Reaction intermediates

A first transitory intermediate. During preliminary experiments, it had been observed that the identified reaction product (di-O-*tert*-butyldimethylsilyl (di-O-TBDMS) styrene) was more difficult to detect when the duration of the reaction in DMF-DMA was too long. Consequently, fenoterol (pure substance) was dissolved in a mixture of DMF-DMA and pyridine (1:5, v/v, 100 μl) and the reaction conducted for only 2 min at 20 °C. A brown colour was immediately observed. A volume of 1 ml of acetonitrile was then added and a brown and viscous residue instantaneously formed in the bottom of the tube. After shaking and clarification, acetonitrile was taken and kept for LC/FRIT-FABMS analysis, while the viscous residue (insoluble in acetonitrile) was put in glycerol (FAB matrix) on the direct probe and analysed on the SX102A detector (FAB +, R ≈ 2500). Calibration was done with Ultramark.

The mass spectrum obtained under these conditions is shown in Fig. 4. A quasi-molecular ion was identified at *m/z* 391 and a structure hypothesis was made. It should correspond to the formula $C_{21}H_{30}O_5N_2$. Although HR confirmations were not performed, some fragmentation proposals are given in Fig. 4. This presumptive intermediate is not stable, since the corresponding components were not identified for other β-agonists which were studied later by means of FABMS experiments. Such a molecule formation would prove that DMF-DMA can react with the secondary amine of the β-agonist ethanolamine side-chain, the alkoxyl groups of DMF-DMA being involved in the reaction. The formation of this kind of intermediate has been mentioned previously for primary sulfonamides,[36] but in that case the reaction involves a primary instead of a secondary amine and the reaction leads to an imine.

A second intermediate: a relatively stable ethanolamide. The acetonitrile-soluble fraction was then analysed by LC/FRIT-FABMS (SX102A, R ≈ 1500). Acetonitrile was evaporated, then taken in the mobile phase, i.e. acetonitrile–water (70:30, v/v) containing 1% of glycerol. The mobile phase flow rate was set at ~0.7 ml min^{-1}. The HPLC system was coupled to the mass spectrometer by a fused-silica capillary (0.22 μm i.d.) which took the sample up to the FRIT-FAB probe located in the ionization chamber (45 °C). A pneumatic splitter set between the HPLC column and the capillary permitted the eluent flow to be divided by a factor of ten. Under these analytical conditions, a single peak could be observed on the chromatograms. The corresponding mass spectrum is presented in Fig. 4. Further, this compound was also analysed by FAB-HRMS (R ≈ 8000, direct probe). The elemental composition corresponding to the $[M + H]^+$ ion ($C_{18}H_{22}O_5N$ (332.1498 u)) was confirmed unambiguously during these analyses. Taking into account the previously mentioned results for the first intermediate, a hypothesis was put forward concerning the formula. HR studies of the main ions observed in the FAB spectrum confirmed this hypothesis. For instance, the ion detected at *m/z* 314.1386 corresponds to $C_{18}H_{20}O_4N$ (314.1392 u), owing to an H_2O loss. Moreover, fragment ions detected at *m/z* 135.0789 and 107.0488 were shown to correspond to $C_9H_{11}O$ and C_7H_7O, respectively.

Confirmation studies. The formation of an ethanolamide intermediate was confirmed for clenbuterol and cimaterol in the FAB + mode, using TLC/MS (SX102). Clenbuterol and cimaterol (pure substances) were dissolved in DMF-DMA–pyridine (1:5, v/v) and heated at 90 °C for 5 h. The reagent was then evaporated to dryness under nitrogen (40 °C) and the dry residue was dissolved in acetonitrile and put on the TLC plate, glycerol being used as the FAB matrix.

Under such analytical conditions, the N-DMAM ethanolamide was detected and identified for cimaterol and clenbuterol (Fig. 5). One can observe on the FAB mass

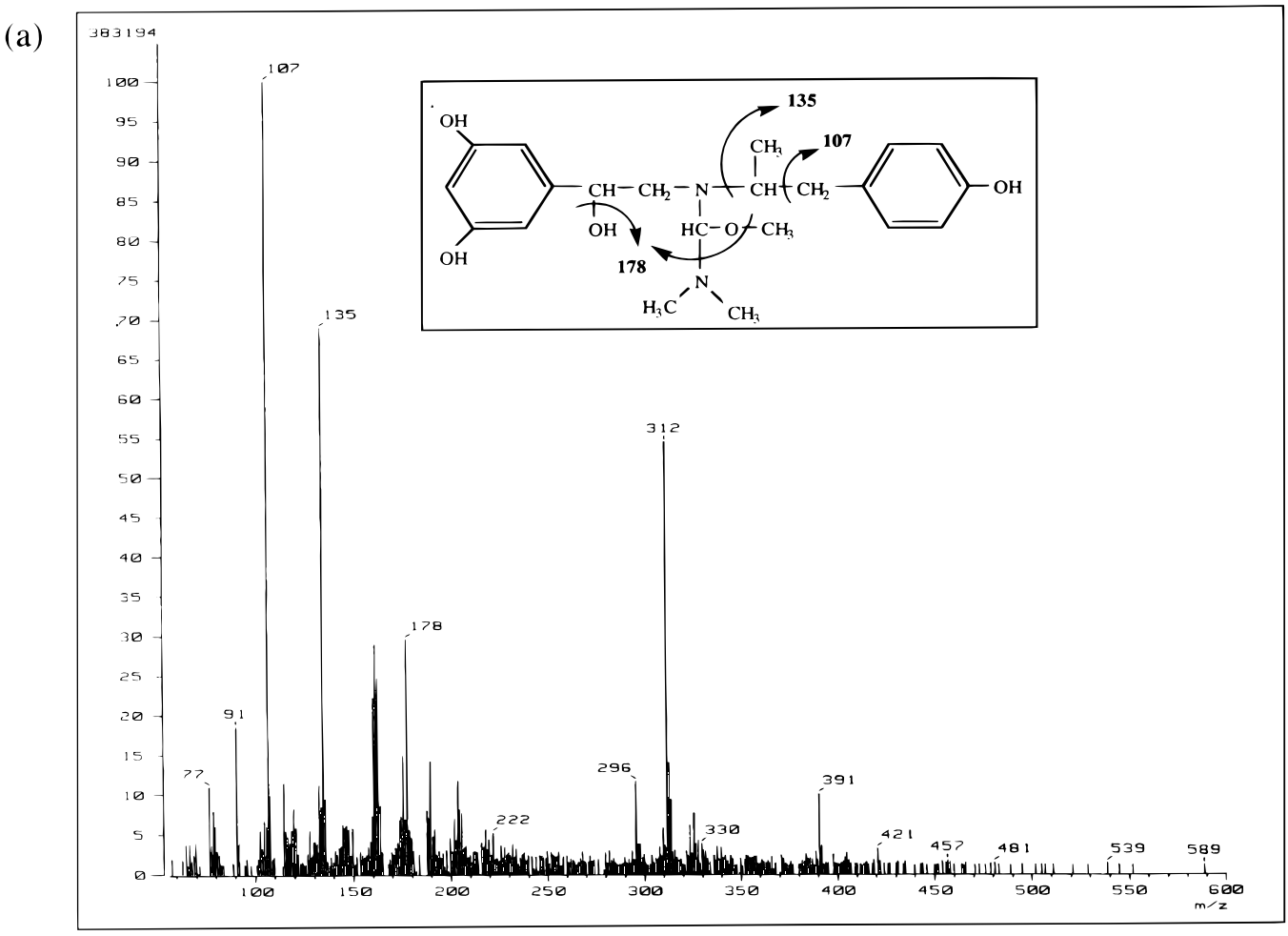


Figure 4. (a) FAB mass spectrum of a first transitory intermediate emphasized for fenoterol via LC/FRIT-FABMS; this compound could not be observed for the other β-agonists studied owing to its high instability, which may be due to its structure close to hemiacetals.

spectrum obtained for cimaterol a quasi-molecular ion at *m/z* 303 and characteristic clusters at $[2M + H]^+$, i.e. *m/z* 605 and $[M + 93]^+$ or $[M + H + glycerol]^+$, i.e. *m/z* 395. For clenbuterol, the quasi-molecular ion can be pointed out at *m/z* 360/362 on the FAB mass spectrum presented in Fig. 5. The above-mentioned results confirm the formation of an ethanolamide intermediate as described previously for fenoterol. On the other hand, the first transitory intermediate (insoluble in acetonitrile) whose formation has been suspected for fenoterol could not be found for the two latter studied molecules.

First steps of the reaction mechanism: condensation of the DMA part of DMF-DMA with the secondary amine to give an ethanolamide intermediate. In the proposed mechanism (Fig. 6), the formation of the first intermediate which occurs secondary to the condensation step of the DMA part of DMF-DMA with the secondary amine of the ethanolamine moiety of β-agonists leads to the elimination of methanol, while the achievement of the ethanolamide intermediate leads to the elimination of trimethylamine. However, the first intermediate (not found for cimaterol or clenbuterol) seems difficult to isolate and identify; this instability could be partly explained by the fact that its structure is close to that of hemiacetals, which are usually difficult (or even impossible) to bring to the fore.

Reaction products stemming from the rupture of the side-chain

Identification. The second reaction product stemming from the rupture of the side-chain then had to be identified. The isolation of such a molecule should actually help us in the understanding of the mechanism involved in the formation of the di-OTBDMS-styrene observed for fenoterol.

Fenoterol (pure substance) was dissolved in DMF-DMA–pyridine (1 : 5, v/v) and heated at 90 °C for 2 min. The vial was then cooled to room temperature and the reagent evaporated to dryness under nitrogen at 40 °C. The dry residue was dissolved in MTBSTFA and heated at 70 °C for 30 min. Preliminary studies were performed by GC/LRMS (SX102A, R ≈ 1500) in the EI ionization mode, using the splitless injection mode (280 °C, 1 min delay).

Two peaks were easily identified; the first eluted corresponds to the component which was looked for, while the second corresponds to the di-OTBDMS-styrene previously reported for fenoterol (Fig. 3). The EI mass

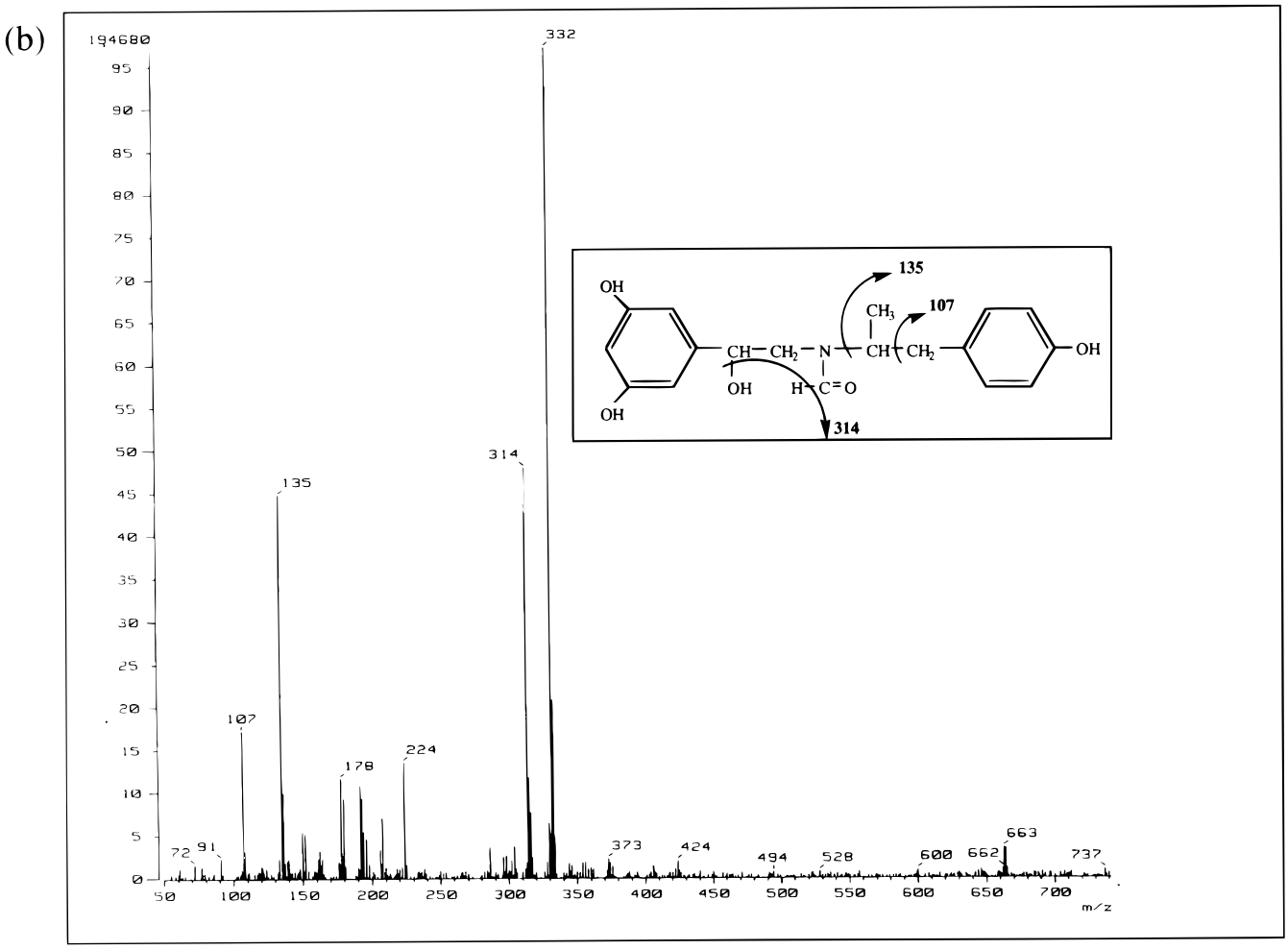


Figure 4. (b) FAB mass spectrum of the ethanolamide intermediate obtained for fenoterol (direct probe), the structure of which was confirmed by means of HR experiments. This intermediate could be observed for each β-agonist studied (see also Fig. 5).

spectrum obtained for the first peak is presented in Fig. 7. An *m/z* 291 molecular ion was confirmed by means of PCI (methane, HP 5971). It was thought that this might be an isocyanate derivative stemming from the ethanolamide. This hypothesis was supported by HR (R $\approx$ 10000) investigations performed under the same conditions. The mass of the molecule detected at *m/z* 291.1667 was found to fit with the formula $C_{16}H_{25}O_2NSi$ (291.1655 u). The elemental analysis of the main ion species detected in the EI mode confirmed such a hypothesis. The mass of the ion detected at *m/z* 221.1329 corresponds to $C_{13}H_{21}OSi$ (i.e. a C_3H_4ON loss when compared with the molecular ion). Moreover, the mass of the fragment ion which was detected at *m/z* 191.0892 corresponds accurately to $C_{11}H_{15}OSi$, such a formula being explained by a $C_5H_{10}ON$ loss when compared with the molecular ion (i.e. the loss of two methyl groups when compared with the $C_{13}H_{21}OSi$ fragment ion). Finally, the mass of the fragment ion detected at *m/z* 234.0916 corresponds to $C_{12}H_{16}O_2NSi$ (i.e. the loss of the *tert*-butyl group of the aromatic ring TBDMS ether). Further, GC/EI-HRMS analyses also confirmed the hypothesis of the styrene formation. The mass of the molecule whose molecular ion was detected at *m/z* 364.2273 corresponds to $C_{20}H_{36}O_2Si_2$ (364.2254 u). The analysis of the elemental composition of the main fragment ions also confirmed the styrene hypothesis. The mass of the ion detected at *m/z* 307.1507 fits well with the formula $C_{16}H_{27}O_2Si_2$ (307.1541 u), i.e. a C_4H_9 loss when compared with the molecular ion. Moreover, the mass of the ion detected at *m/z* 265.1041 would appear to correspond to the formula $C_{13}H_{21}O_2Si_2$, which means a C_7H_{15} loss when compared with the molecular ion. This could be explained by the simultaneous loss of a *tert*-butyl, a methyl and the styrene vinyl groups.

Confirmation studies. These hypotheses were confirmed on several other β-agonists using various MS experiments.

Isocyanates(GC/LRMS, Ross injector). A Ross injector was adapted to the GC/MS SX102 instrument in order to identify the isocyanate reaction products stemming from clenbuterol-like β-agonists. These investigations could not be performed with a split/splitless injector since the expected molecule (being eluted very early on the capillary column) was lost in the solvent head.

Clenbuterol, whose side-chain secondary amine is substituted with a *tert*-butyl group, was studied under such conditions in GC/LRMS (R = 1500) in the EI and PCI (isobutane) modes. Clenbuterol (pure substance) was dissolved in DMF-DMA–pyridine (1:5, v/v) and heated at 90 °C for 5 h. The vial was then cooled to

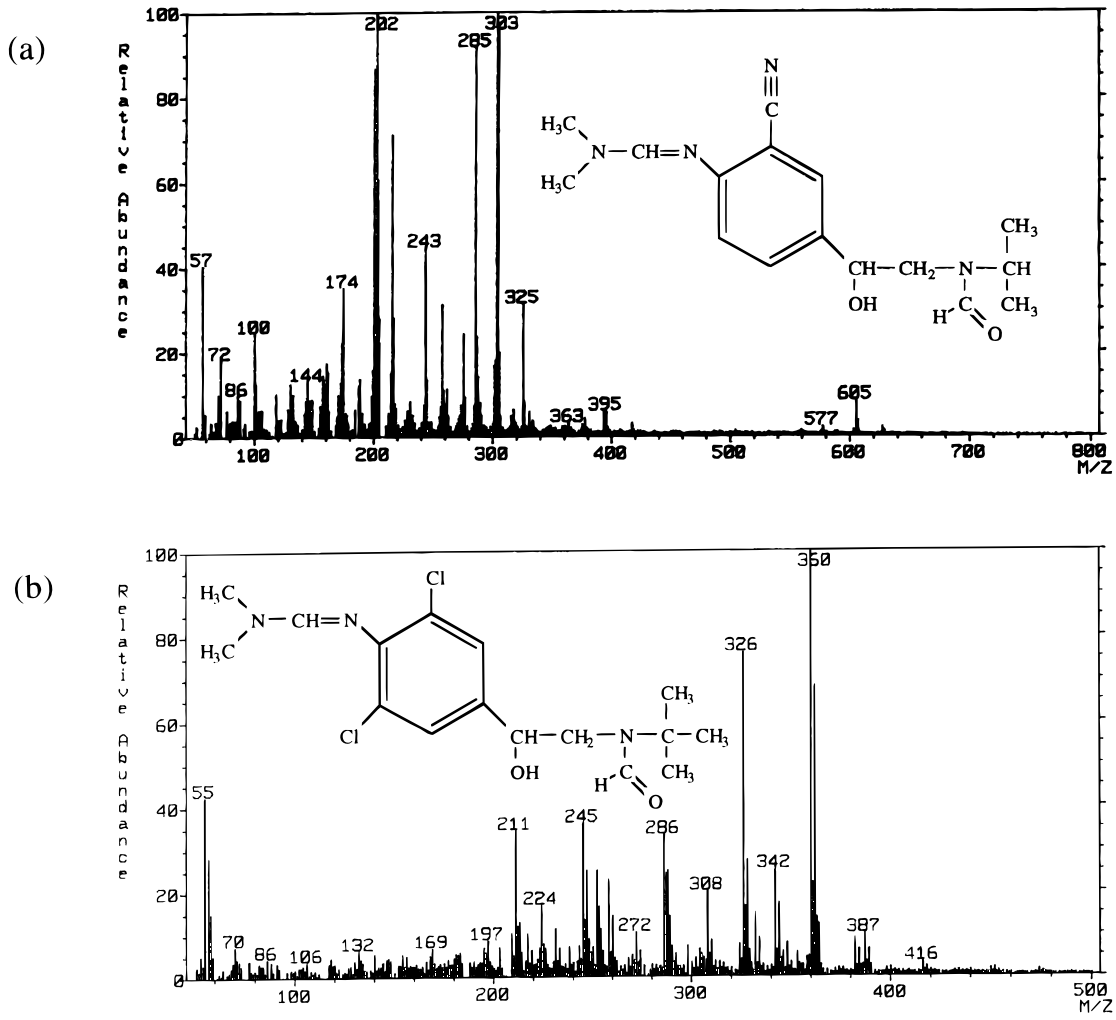


Figure 5. (a) FAB mass spectrum of the ethanolamide intermediate stemming from cimaterol (TLC/MS investigation); note the quasi-molecular ion at m/z 303 and characteristic clusters at $[2M+H]^+$, i.e. m/z 605 and $[M+H+glycerol]^+$, i.e. m/z 395. (b) FAB mass spectrum of the ethanolamide intermediate stemming from clenbuterol (TLC/MS investigation); note the quasi-molecular ion at m/z 360.

room temperature and the reagent evaporated to dryness under nitrogen at 40 °C. The dry residue was dissolved in 50 µl of acetonitrile and a 2 µl aliquot was put on the Ross injector needle. The EI and PCI mass spectra obtained under such conditions for the *tert*-butyl isocyanate (which was eluted at ~1.5 min) were easily identified.

Styrenes (GC/HRMS, split/splitless injector). GC/EIMS analyses were also performed at a resolution of ~10 000 on the SX102A apparatus. The analytical conditions were identical with those described above, except for the injection mode, as a split/splitless (280 °C) injector was used in the present case (splitless, 1 min delay). With regard to clenbuterol (Fig. 3), HR analyses made possible the confirmation of the main fragment ions observed on the EI mass spectrum of the N-DMAM-styrene. The mass of the molecular ion which was detected at *m/z* 242.0401 was found to correspond to the formula $C_{11}H_{12}N_2^{35}Cl_2$ (242.0378 u). Further, the isotopic contribution detected at *m/z* 244.0348 corresponds accurately to the expected formula. The mass of the main fragment ion (detected at *m/z* 207.0688) corresponds to $C_{11}H_{12}N_2^{35}Cl$ (207.0689 u). This main ion species (base peak) can therefore be explained by the loss of a chlorine atom. This loss was confirmed with the detection at *m/z* 209.0710 of the isotopic contribution stemming from ^{37}Cl (209.0660 u). The loss of a chlorine atom was also emphasized for β-agonists structurally close to clenbuterol (such as mabuterol, for instance).

Concerning cimaterol, preliminary HR ($R \approx 10\,000$) investigations enabled us to confirm the elemental formula of the molecular ion, i.e. $C_{12}H_{13}N_3$ (199.1109 u). On the other hand, such a resolution could not permit the accurate identification of the main fragment ion observed on the EI mass spectrum (Fig. 8). This fragment could actually stem from the loss of both methyl and vinyl groups leading to a $C_9H_7N_3$ (157.0640 u) fragment or from a C_2H_4N loss (leading to a $C_{10}H_9N_2$ fragment, 157.0761 u), this last explanation being *a priori* more difficult to understand. Consequently, MS investigations were performed at a resolution of 20 000 in the SIM acquisition mode in order to rule in or rule out these hypotheses. The results clearly revealed a C_2H_4N loss leading to an *m/z* 157.0761 instead of an *m/z* 157.0640 fragment. The main fragmentation pattern obtained for the N-DMAM styrene of cimaterol is presented in Fig. 9.

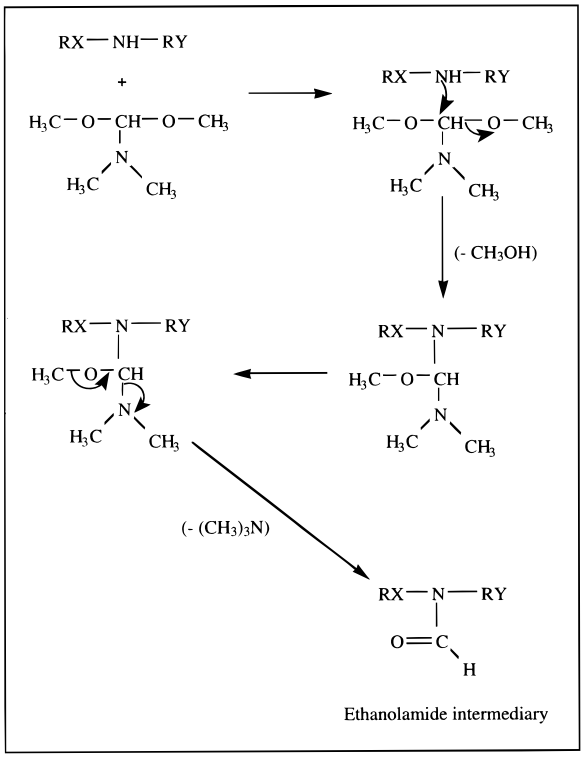


Figure 6. Schematic depiction of reaction mechanisms leading from the ethanolamine moiety of β-agonists to the ethanolamide intermediate via condensation of the DMA part of DMF-DMA and elimination of methanol (as a first step), then trimethylamine (as a second step).

The results of EI-HR full-scan analysis of such a molecule were as follows:

m/z 199.1089, $C_{12}H_{13}N_3$ (199.1109 u)
m/z 184.0865, $C_{11}H_{10}N_3$ (184.0875 u)
m/z 171.0914, $C_{11}H_{11}N_2$ (171.0922 u)
m/z 157.0761, $C_{10}H_9N_2$ (157.0761 u)
m/z 155.0610, $C_{10}H_7N_2$ (155.0610 u)
m/z 129.0581, C_9H_7N (129.0579 u).

The EI fragmentation leading to the $C_{10}H_9N_2$ fragment ion of the N-DMAM-styrene stemming from cimaterol is also encountered for clenbuterol. In this case, a C_2H_4N loss (instead of the loss of both a methyl and a vinyl group, as expected) was also proved. A resolution of 15 870 was theoretically necessary to distinguish the corresponding masses (200.0034 and 199.9908 u, respectively); nevertheless, a resolution of 30 000 had to be used to control the total lack of response at *m/z* 199.9908 via HRSIM.

Reaction mechanism. Previous results revealed that the two components observed by means of GC/MS (styrene and isocyanate) partly depend on a dehydration step of the ethanolamide intermediate. FAB analyses being performed at low temperature, and since these derivatives could not be observed with FAB analyses whatever the introduction mode, it was assumed that this dehydration step was highly dependent upon temperature. Consequently, further experiments had to be carried out in order to control the influence of temperature on the reaction mechanism and to look for a potential third intermediate, whose side-chain would not be broken up.

A third reaction product which does not involve rupture of the side-chain

As a third compound (reaction intermediate leading to the styrene and isocyanate derivatives?) stemming from a dehydration step of the ethanolamide was suspected, chromatograms were carefully analysed. Compounds eluting late from the capillary column were examined for the studied β-agonists. Components observed for clenbuterol and NA1141 (hydroxymethylclenbuterol) were examined more precisely. The identification of the compounds stemming from these two β-agonists was actually easier, owing to the chlorine-containing aromatic rings.

GC/MS (clenbuterol). Analyses were performed on the SX102 GC/MS apparatus, equipped with a Ross injector (280 °C) (R $\approx$ 1500). Clenbuterol (pure substance) was dissolved in DMF-DMA–pyridine (1:5, v/v) and heated for 20 min at 90 °C. The vial was cooled to room temperature, the reagent was evaporated to dryness under nitrogen (40 °C) and the dry residue was dissolved in 50 µl of acetonitrile. A 2 µl aliquot was finally put on the glass needle. The EI mass spectrum obtained for the compound which was looked for is shown in Fig. 10. A structural hypothesis was then made; it would appear to be a 2-one substituted azetidine with an *m/z* 341 molecular ion. These results were confirmed via PCI (200 eV, isobutane). The previously mentioned reaction conditions enabled us to observe, besides the *tert*-butyl isocyanate, the styrene and azetidine derivatives whose primary amine is not substituted with the DMAM group (owing to an incomplete reaction).

Linked-scan confirmatory experiment (NA1141). When treated with DMF-DMA, NA1141 gives the same N-DMAM-styrene as that observed for clenbuterol (molecular ion set at *m/z* 242). Further, an *m/z* 242 fragment ion can also be observed in the EI mass spectrum of the N-DMAM-azetidine stemming from NA1141. Consequently, these ion species were studied in the linked-scan acquisition mode on the SX102A GC/MS system (EI, fragment ions, splitless, 1 min delay (280 °C), PFK calibration, R $\approx$ 1500). For these experiments, NA1141 samples in DMF-DMA–pyridine were heated at 90 °C for 5 h.

The results obtained are presented in Fig. 11. As the fragment ions stemming from *m/z* 242 were identical for the N-DMAM-styrene (retention time (RT) 13 min) and for the N-DMAM-azetidine (RT 25 min), it was concluded that the styrene molecular ion is structurally strictly identical with the corresponding azetidine fragment ion. An analogy between a chemical mechanism (loss of the side-chain due to DMF-DMA and heat) and a physico-chemical phenomenon (EI fragmentation of the azetidine) can therefore be suggested.

Nevertheless, such an analogy could not let us conclude that azetidine derivatives might constitute reaction intermediates leading to the styrene and isocyanate reaction products. The nature of azetidine derivatives (intermediates or reaction products?) therefore had to be identified. For this purpose, the influence of temperature on the different steps of the reaction mechanism was studied.

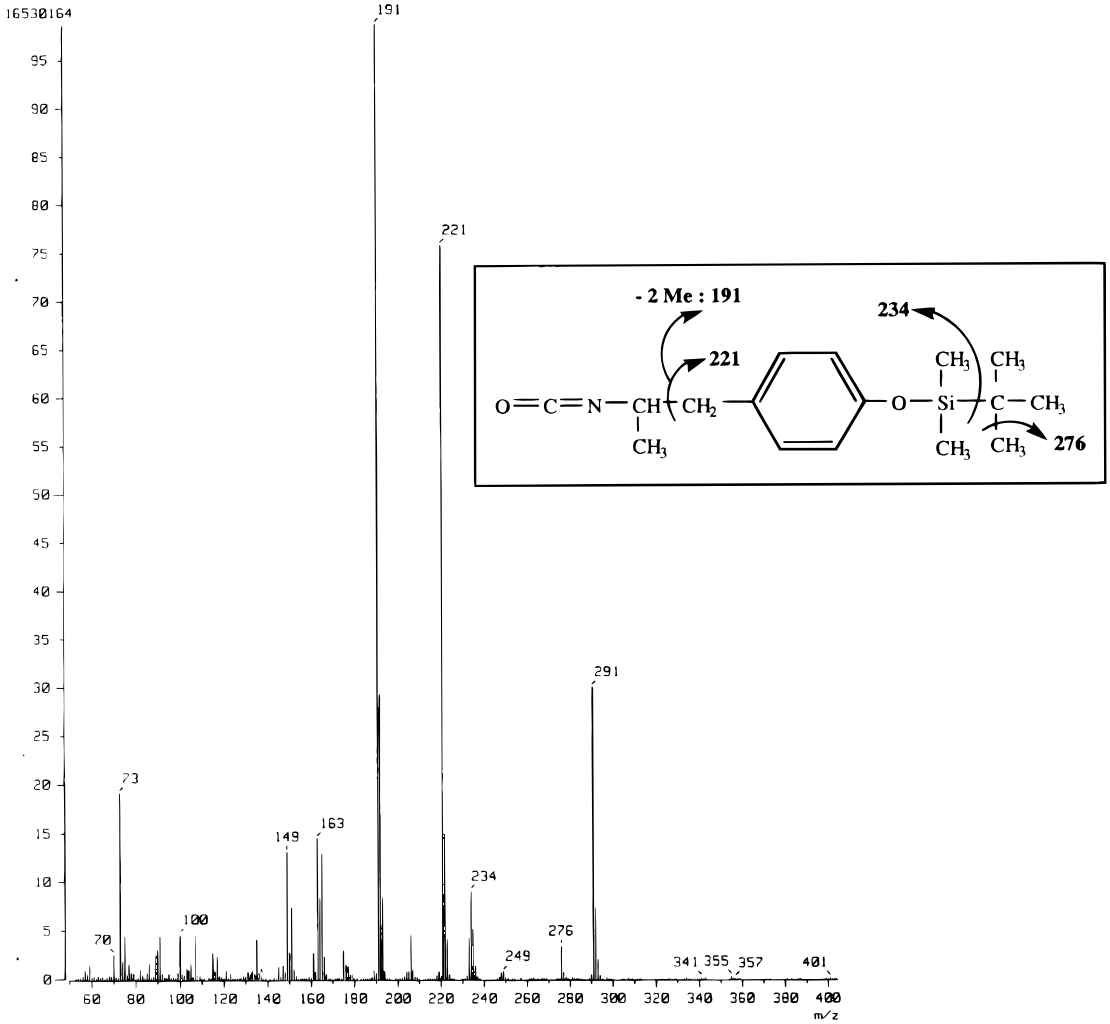


Figure 7. EI mass spectrum of the O-TBDMS isocyanate derivative stemming from fenoterol, i.e. a β-agonist which exhibits a heavy phenol-containing side-chain (GC/MS, injection in the splitless mode, 280 °C). Note the molecular ion at m/z 291 and the large number of fragment ions to be used for identification purposes.

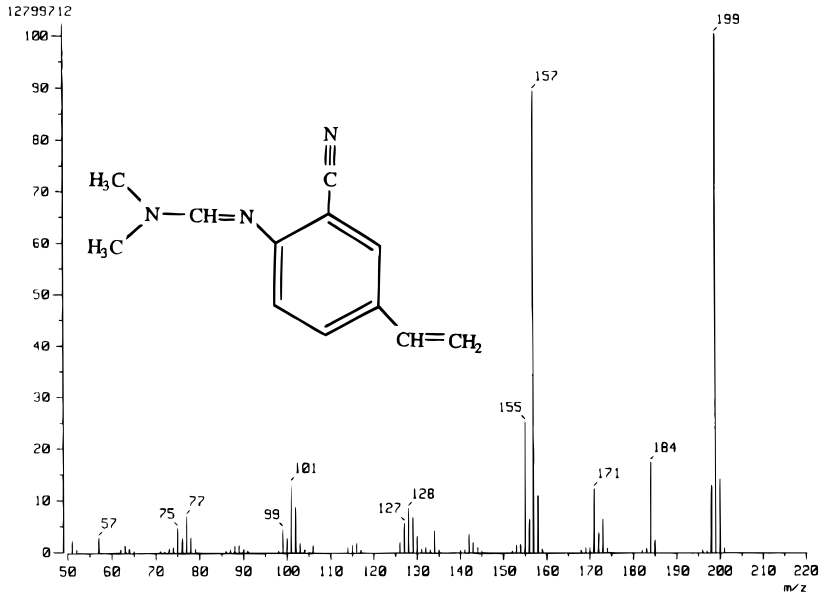


Figure 8. EI mass spectrum of the N-DMAM-styrene derivative stemming from cimaterol (GC/MS).

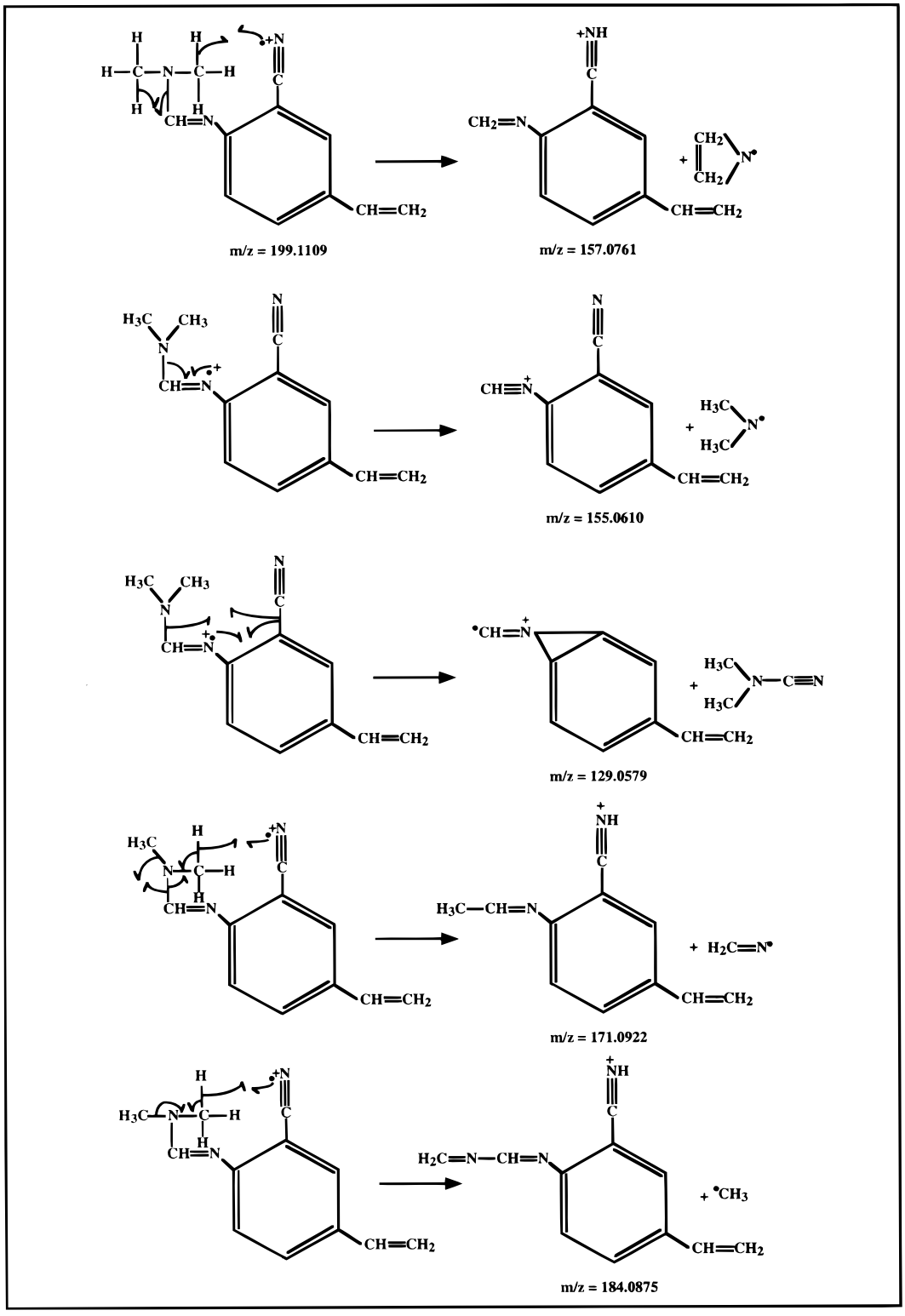


Figure 9. Main fragmentation pattern observed for the N-DMAM-styrene stemming from cimaterol via GC/EIMS analyses; as a result of EI-HRMS full-scan analyses (R ≈ 20 000).

Influence of temperature

Duration of high-temperature exposures. The behaviour of different β-agonistic drugs when treated with DMF-DMA and exposed to high temperature (90 °C) was studied on a Model 5890–5971 GC/EIMS quadrupolar system.

β-Agonists free of hydroxy group(s) on the aromatic ring (clenbuterol, clenpenterol, NA1141, tulobuterol, mabuterol, mapenterol, cimaterol, cimbuterol) were treated as follows. For each β-agonist, 120 µl of a 100 ng μl^{-1} methanol standard solution were evaporated to dryness under nitrogen (40 °C) and 50 µl of pyridine and 10 µl of DMF-DMA were added to the dry residue.

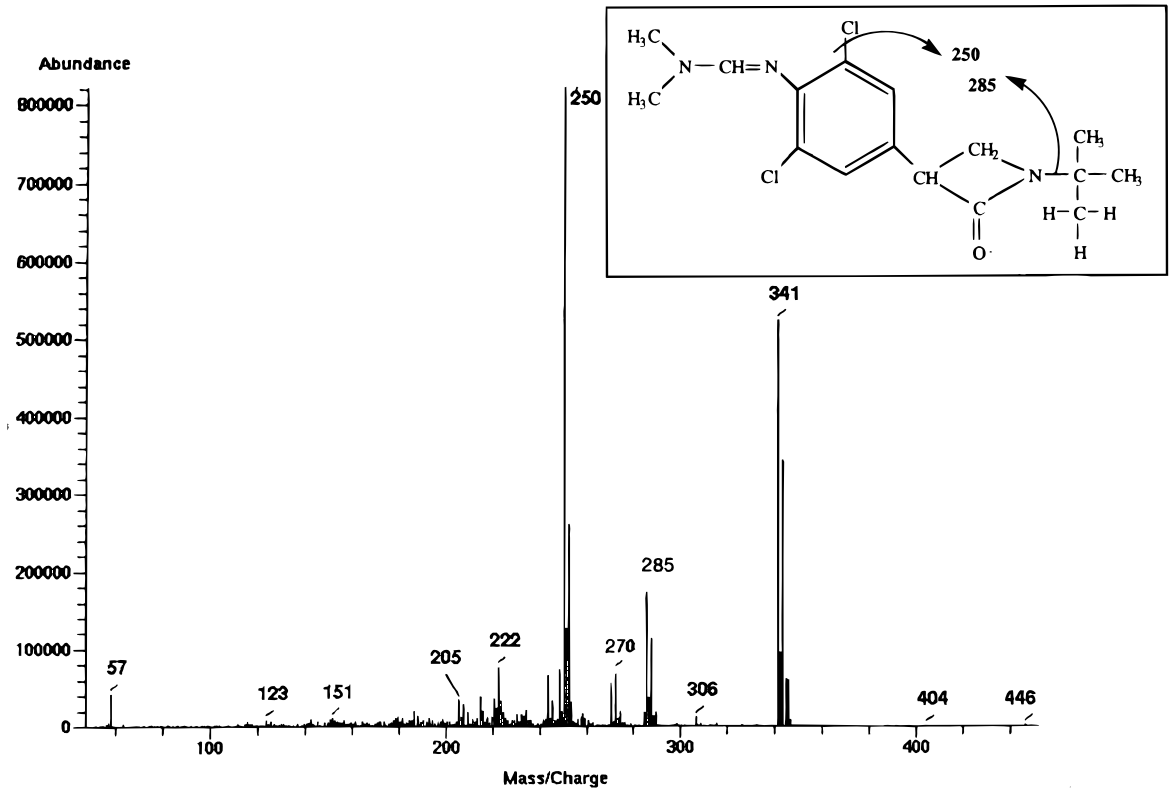


Figure 10. EI mass spectrum of the azetidine derivative stemming from clenbuterol. Note the molecular ion at m/z 341 and the loss of a chlorine atom (leading to m/z 250) which can be observed for every chlorine-containing β-agonist.

After vortex mixing, 1 µl was directly injected and analysed via GC/EIMS; the vials were then heated at 90 °C for 2, 30, 60 and 90 min and 2, 3, 5 and 20 h before injection.

β-Agonists containing hydroxy group(s) on the aromatic ring (fenoterol, terbutaline, ractopamine) were derivatized as follows. For each β-agonist, 80 µl of a 100 ng μl^{-1} methanol standard solution were evaporated to dryness under nitrogen (40 °C) and 25 µl of pyridine, 5 µl of DMF-DMA and 10 µl of MTBSTFA were added to the dry residue. After vortex mixing, 1 µl was directly injected and analysed via GC/EIMS; the vials were then heated at 90 °C for 2, 30, 60 and 90 min and 2, 3, 5 and 20 h before injection.

Analyses were performed in the full-scan acquisition mode. Heights of base peaks of styrene (N-DMAM or mono- or di-O-TBDMS) EI mass spectra were used to follow the reaction kinetics. The results which were obtained for this experiment are presented in Fig. 12.

It has been observed that a long reaction time is suitable for the main compounds of the first group (free of hydroxy group(s)) and that an optimal response can be observed for a reaction duration close to 5 h. On the other hand, terbutaline, ractopamine and fenoterol would appear to react instantaneously. When performed at room temperature, similar results were obtained, except for the duration of reaction, which had to be prolonged for clenbuterol-like compounds. Azetidine derivatives were also studied; they were found to evolve as styrene derivatives. This behaviour made us believe that styrene and isocyanate derivatives would not stem from the corresponding azetidine.

The results in Fig. 12 might indicate that the ethanolamide formation depends on the duration of reaction for most β-agonists, except hydroxylated aromatic ring containing compounds. The kinetics obtained for tulobuterol (a β-agonist which is free of primary amine on its aromatic ring), being identical with N-DMAM-styrenes, reveals that the improvement of the response with the duration of reaction cannot be explained by the gradual formation of N-DMAM derivatives which are known to be obtained at high temperatures. Nevertheless, an LC/FRIT-FABMS study would be necessary to confirm this hypothesis via the direct study of the ethanolamide formation.

We then had to demonstrate that the rupture of the side-chain leading to the styrene and isocyanate derivatives, and the formation of azetidine derivatives, depend partly on the high temperatures used in the injection port.

Injector temperature. A study of the influence of the injector temperature was performed on the SX102 GC/MS apparatus, equipped with a Ross injector. These experiments were performed on clenbuterol (pure substance derivatized in DMF-DMA–pyridine (1 : 5, v/v) for 5 h at 90 °C). After cooling to room temperature, samples were analysed via GC/EIMS in the full-scan mode. Three injection temperatures (150, 200 and 280 °C) were tested. The corresponding results are reported in Fig.13. It can be seen that the responses observed for the N-DMAM-styrene and the *tert*-butyl isocyanate, and also the N-DMAM-azetidine, are improved with the highest temperature used. This led us to believe that the three

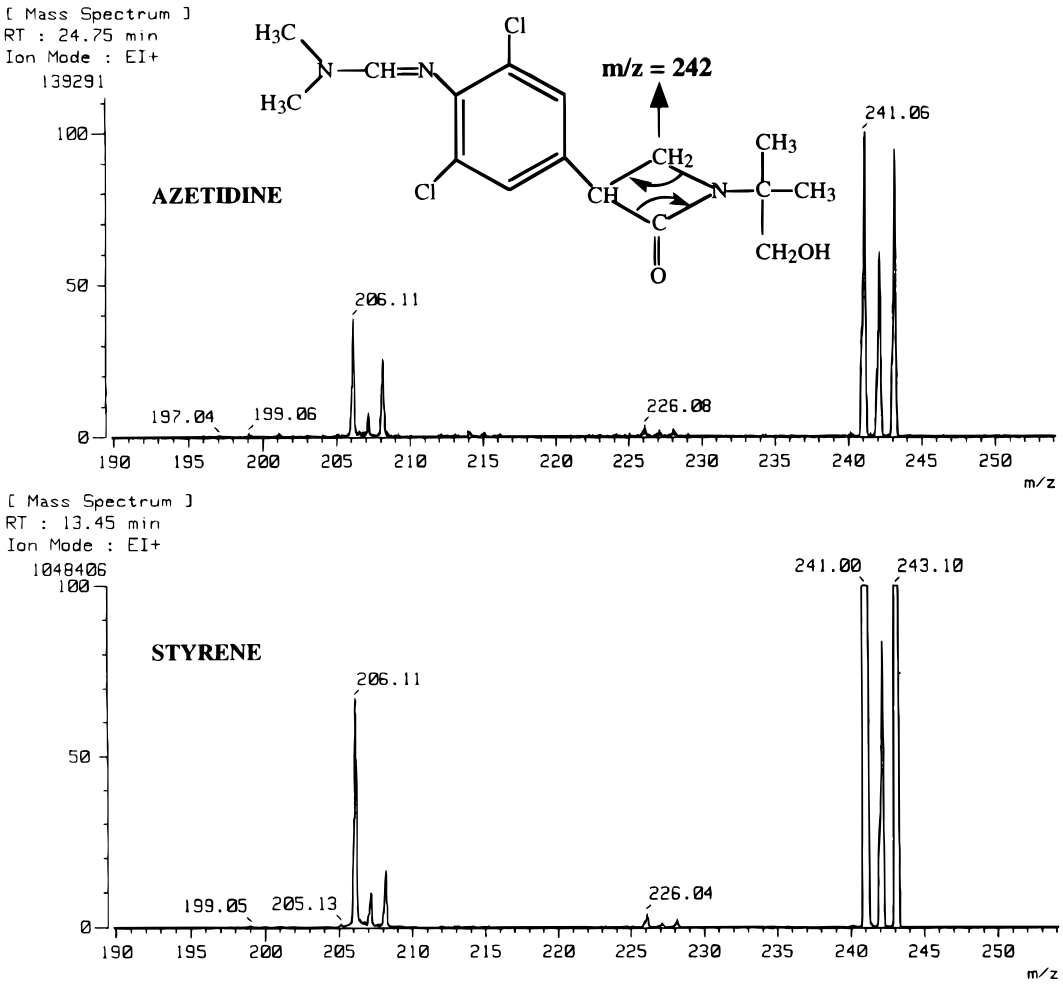


Figure 11. Result of a linked-scan experiment (GC/EIMS, fragment ions) which demonstrates that the molecular ion of the N-DMAM-styrene derivative stemming from NA1141 (m/z 242) is strictly identical with the fragment ion observed in the corresponding N-DMAM-azetidine derivative EI mass spectrum.

components identified when GC/MS is used stem from the high temperatures of the injector which makes the dehydration of the ethanolamide possible. Such a similarity of behaviour would appear to invalidate the hypothesis (however conceivable) that the azetidine derivative could be a reaction intermediate leading to the isocyanate and styrene derivatives. Therefore, the ethanolamide intermediate appeared to be a main component of the reaction mechanism.

DEI study. DEI analyses based on cimaterol were also carried out on the SX102 mass spectrometer using a following programming rate of 1/16 A min^{-1} from 0 to 370 mA (2 min), then 2 A min^{-1} from 370 to 500 mA. It can be seen from the results obtained under these conditions that the selective desorption of the N-DMAM-styrene derivative occurs at a higher temperature than that required for the N-DMAM-ethanolamide (Fig. 14).

Elucidation of reaction mechanisms

The results of the different experiments which were devoted to understanding the reaction mechanism are summarized in Fig. 15. Concerning the ethanolamide intermediate formation, the influence of the reaction conditions remains questionable, since LC/FRIT-FABMS analyses were not performed. On the other hand, the reaction steps which lead from the ethanolamide intermediate to the three reaction products are highly dependent on high temperatures for each studied β-agonist (clenbuterol-like and terbutaline-like molecules), styrene, isocyanate and azetidine derivatives being isolated by means of GC/MS or DEI experiments. Further, styrenes stemming from terbutaline-like compounds were proved to be unstable when the duration of reaction at 90 °C is too long, thus leading to the hypothesis that secondary products (polymers?) might form.

Particular case of salbutamol

However, the results reported here could not be directly generalized to a particular β-agonist. Actually, the expected reaction products could not be obtained (or only in small amounts) for salbutamol by means of GC/MS analyses. Specific phenomena were suspected and studied. The problems which were encountered during this experiment were due to the fact that BSTFA was used to derivatize free hydroxy groups of salbutamol, once derivatized with DMF-DMA. Detailed

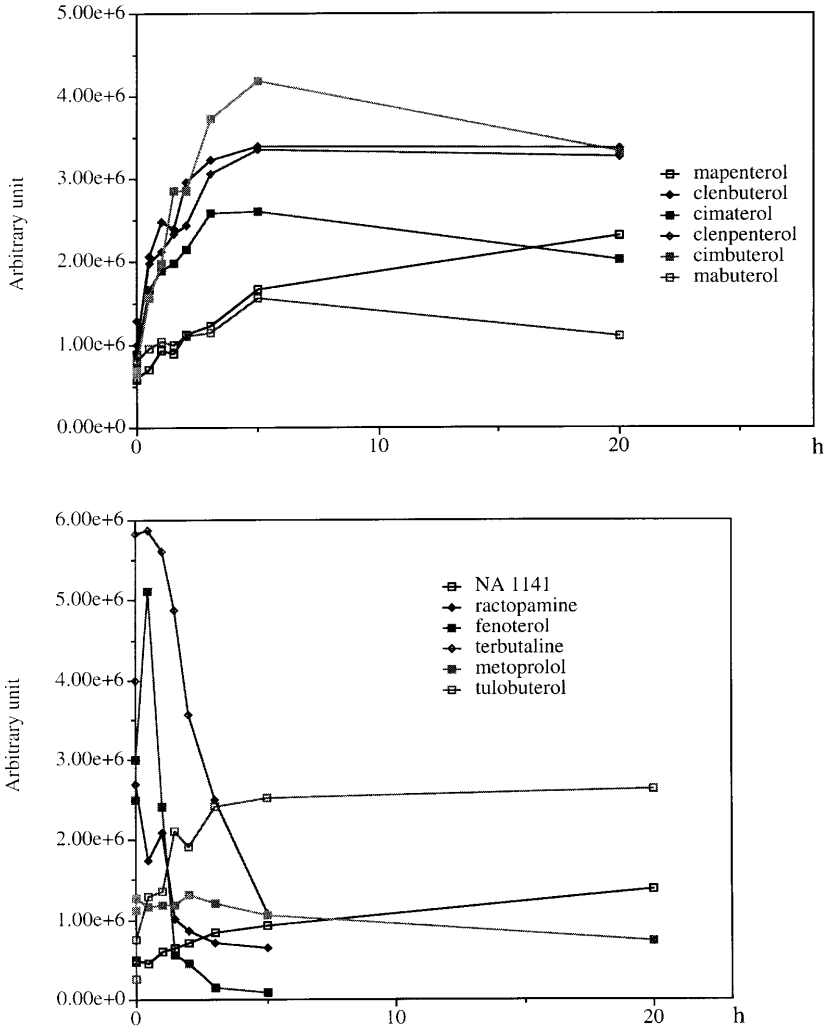


Figure 12. Influence of the duration of reaction in DMF-DMA at 90 °C on styrene formation for the 11 β-agonists studied and a β-blocking agent (metoprolol). GC/EIMS results based on heights of base peak measurements (arbitrary units) plotted against the duration of reaction (h). A long time of reaction (close to 5 h) is suitable for most β-agonists (free of hydroxy groups on the aromatic ring), while terbutaline, ractopamine and fenoterol would appear to react instantaneously.

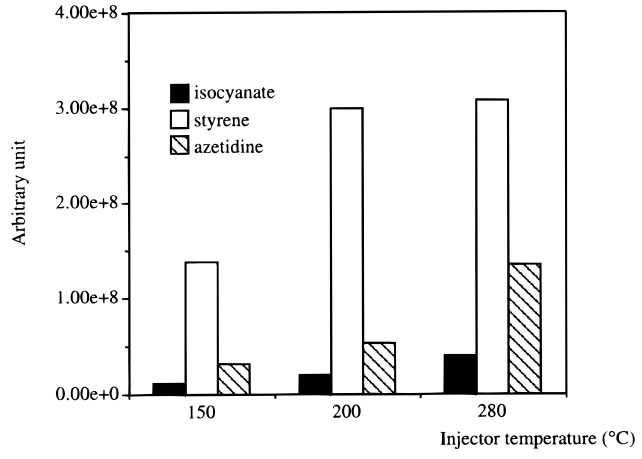


Figure 13. Influence of the temperature of the Ross injector on the styrene, isocyanate and azetidine derivatives. Heights of peaks (TIC, n = 3) obtained for the N-DMAM-styrene and -azetidine and for the *tert*-butyl isocyanate stemming from clenbuterol (arbitrary units) were plotted against the temperature of the injector. One can observe that the response obtained for the three derivatives is improved with the highest temperature.

experiments performed with BSTFA have been reviewed.[38] In spite of a lack of convincing results with such a silylation reagent, these preliminary investigations led to the conclusion that the primary alcohol of the aromatic ring should react with DMF-DMA in a specific way. These preliminary results have shown that DMF-DMA reacts with the primary alcohol group of salbutamol aromatic ring to give a dimethylamino group.[38] Experiments were then performed using MTBSTFA as the silylating reagent. The dry residue of salbutamol (pure substance) was taken in 50 µl of DMF-DMA–pyridine (1:5, v/v) and heated at 90 °C for 5 h. After cooling, the reagent mixture was evaporated to dryness under nitrogen (40 °C) and the dry residue was dissolved in 30 µl of MTBSTFA and heated at 70 °C for 60 min, prior to GC/MS analysis. Under such conditions, the main components obtained (apart from the *tert*-butyl isocyanate) are presented in Fig. 16. They correspond to the unexpected styrene and azetidine derivatives characterized by a dimethylaminomethyl- and O-TBDMS-containing aromatic ring.

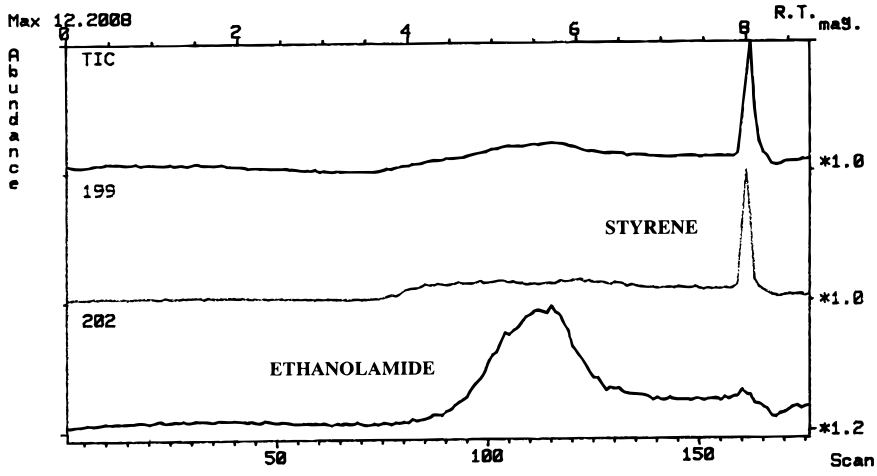


Figure 14. Chromatographic results of the DEI study. Note the selective desorption of the N-DMAM-styrene derivative (stemming in the present case from cimaterol) which occurs at a higher temperature than that required for the N-DMAM-azetidine.

Apart from the loss of a methyl group (*m/z* 276), one can see from the styrene EI mass spectrum a fragment ion detected at *m/z* 243 which corresponds to the loss of the TBDMS *tert*-butyl group. Among the large number of fragment ions which can be observed in the azetidine EI mass spectrum, a similar kind of loss can be recognised. Moreover, the nature of molecular ions and of the main fragment ions were confirmed via HRSIM

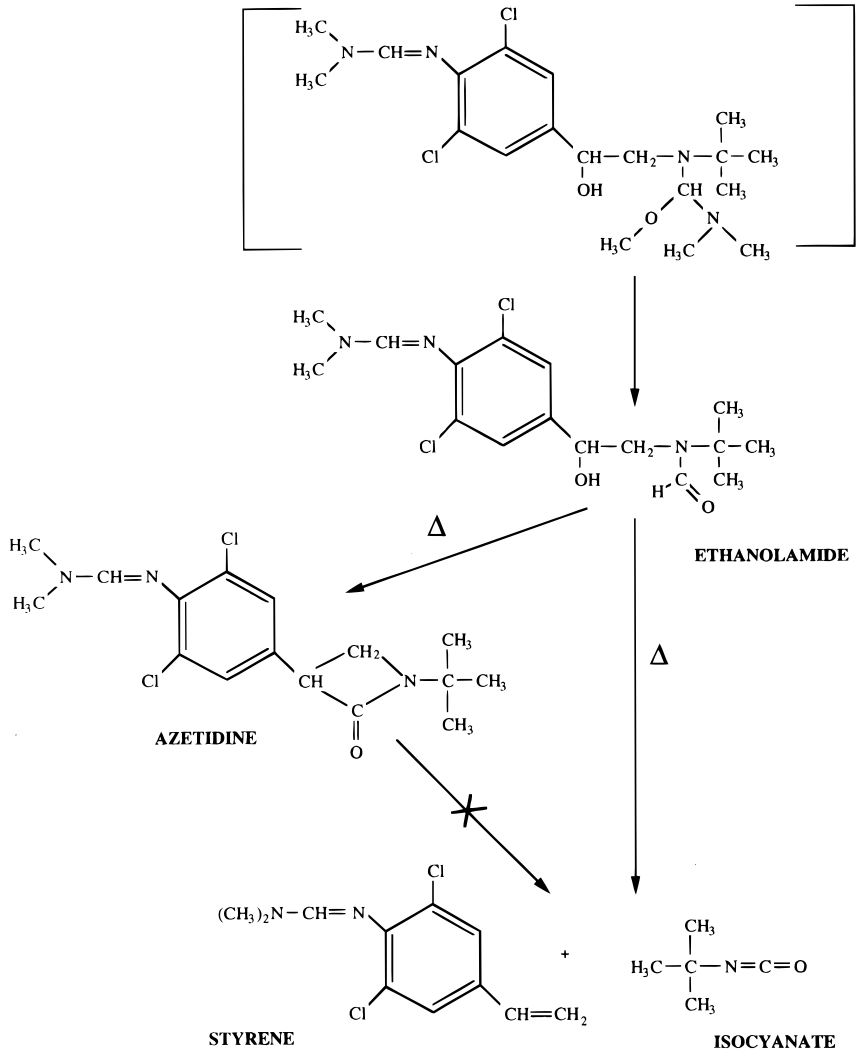


Figure 15. Schematic depiction of the main components (intermediates and reaction products) obtained during the specific reaction which occurs between DMF-DMA and the ethanolamine moiety of β-agonists; example of clenbuterol. The formation of the three identified reaction products (styrene, isocyanate and azetidine) was shown to be dependent on high temperatures, which lead to the dehydration of the ethanolamide intermediate.

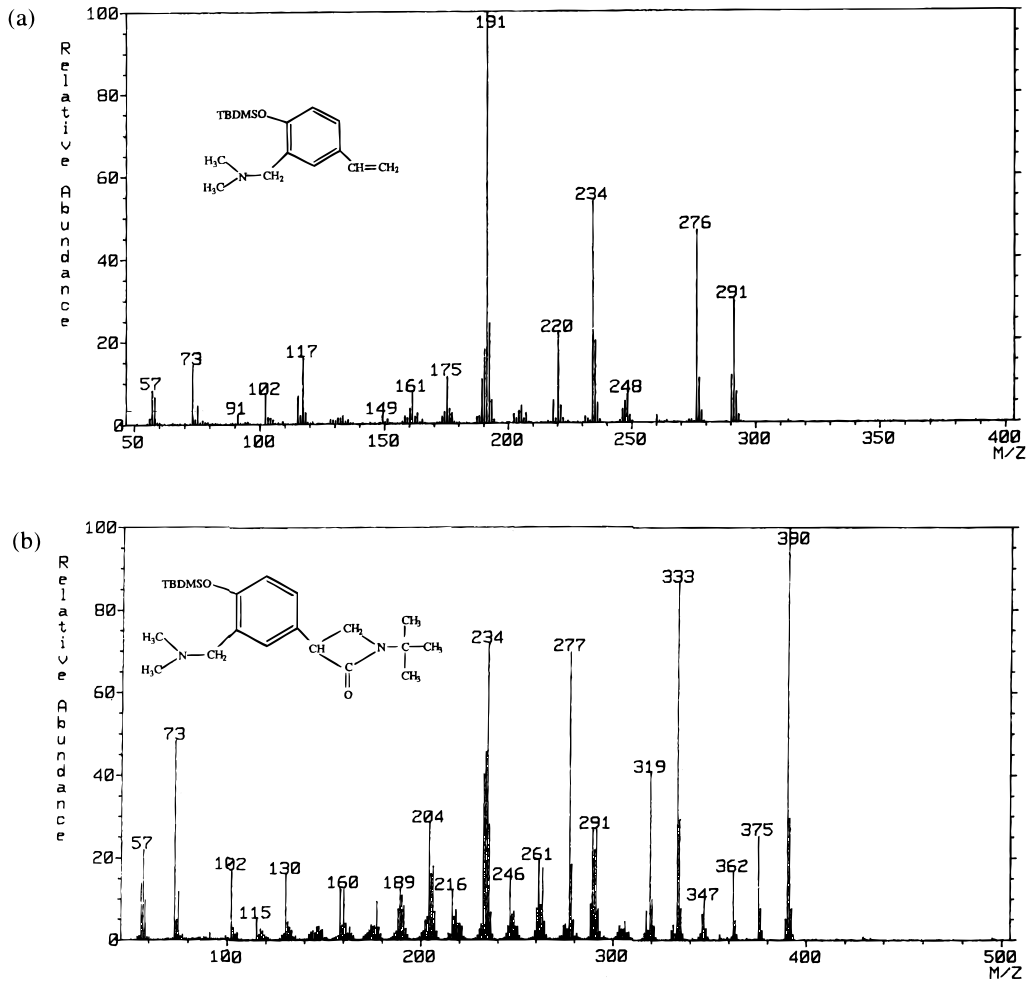


Figure 16. Particular case of salbutamol. (a) EI mass spectrum of the dimethylaminomethyl O-TBDMS-substituted styrene stemming from salbutamol (GC/MS). (b) Corresponding azetidine EI mass spectrum (GC/MS).

($R \approx 10\,000$). Once again (when compared with previous linked-scan experiments), a fragment ion rigorously identical with the molecular ion of the styrene derivative has been identified at 291.2018 u (C_5H_9ON loss) in the azetidine mass spectrum.

Further, as a preliminary result, the addition of DCC to the DMF-DMA mixture would appear to catalyse the azetidine formation, the secondary alcohol of the side-chain being able to react with DCCH to catalyse the dehydration of the ethanolamide, leading in that case to the azetidine derivative and an N,N′-dicyclohexylurea molecule. This last observation could be helpful when one attempts to enhance azetidine formation for confirmatory purposes.

Practical interest

The understanding of reaction mechanisms would appear to offer valuable analytical solutions. They are attractive for the analyst since analytical methods can be designed for either screening for a large number of β-agonists (GC/MS analysis of styrene derivatives) or confirming the illegal use of a particular β-agonist in meat-producing animals (GC/MS analysis of styrene, isocyanate and azetidine derivatives using a Ross injector and/or TLC or LC/MS of the ethanolamide intermediate). For screening purposes, it is better to perform two distinct investigations, one being devoted to clenbuterol-like compounds and salbutamol (at least 5 h in the DMF-DMA mixture) and the other to terbutaline-like compounds (flash reaction). For confirmatory purposes, the knowledge of reaction mechanisms would also appear to be extremely helpful when combined with various analytical techniques. As a consequence, the use of DMF-DMA makes the SIM acquisition process easier, while improving its practicability, especially when magnetic sectors are used for the detection at a resolution higher than 5000. Preliminary experiments were performed in order to test the sensitivity of this technique. Blank calf urines were spiked with 1 ng ml^{-1} of clenbuterol, cimaterol, mabuterol or terbutaline. Metoprolol (β-blocking agent) was added at the 5 ng ml^{-1} level and used as the internal standard for clenbuterol-, cimaterol- and mabuterol-containing urines. Urines were extracted as described previously[19] and analysed by means of GC/EI-LRMS (SIM mode) on a quadrupolar instrument, clenbuterol, mabuterol and cimaterol being derivatized according to the clenbuterol-like technique and terbutaline according to the terbutaline-like technique. The results which were obtained in terms of signal-to-noise ratio are presented in Table 1. Real calf urine samples stemming from treated animals were also analysed successfully; the less

Table 1. Peak-to-peak signal-to-noise ratio obtained from calf urine samples spiked with 1 ng ml^{-1} of mabuterol, clenbuterol, cimaterol and terbutaline and 5 ng ml^{-1} of metoprolol (styrene derivatives, $n = 5$) (GC/EI-LRMS, injection in the splitless mode at 280 °C)

Compound	Mean[a]	SD[b]	RSD[c] (%)
Mabuterol (*m/z* 241)	45.2	7.45	16.5
Clenbuterol (*m/z* 242)	30.6	4.35	14.2
Cimaterol (*m/z* 199)	58.2	5.92	10.2
Terbutaline (*m/z* 307)	49.2	4.77	9.7
Metoprolol (*m/z* 147)	71.7	4.83	6.7

[a] Mean values.
[b] Standard deviation.
[c] Relative standard deviation.

concentrated ones were analysed via GC/EI-HRMS (SIM mode). An example of the signal which was obtained with a resolution close to 10 000 for a sample containing 40 pg ml^{-1} of clenbuterol is shown in Fig. 17.

The sensitivity of this technique looks promising but further experiments (using DCC?) would be necessary to enhance the formation of azetidine derivatives in low-concentration samples.

DISCUSSION

The specific reaction studied here has not been described previously and should be extended in further experiments to other secondary ethanolamines. Divisions of molecules have been observed by Scoggins[39] in the particular case of the methylation of amino groups, but such phenomena have not been reported previously for DMF-DMA, except in the study of Rüttimann *et al.*, which describes the decarboxylation of δ-hydroxycyclohexene-β,γ-unsaturated carboxylic acids in the presence of DMF-DMA and an apolar solvent.[40] The rupture of a molecule usually leads a reagent and/or an analytical procedure being discarded. However, the rupture of the ethanolamine side-chain observed in the present study has appeared as a major tool in the field of control of illegally used β-agonists. It has actually been shown that the ethanolamide which formed prior to the rupture was a relatively stable component which leads after a dehydration step to three reaction products, all (ethanolamide and reaction products) being suitable for use for identification purposes. Further, a single ion stemming from styrene and azetidine derivatives can be used for confirmation purposes via linked-scan or HRSIM analyses. The better understanding of the reaction should therefore allow us to define an analytical strategy based on DMF-DMA

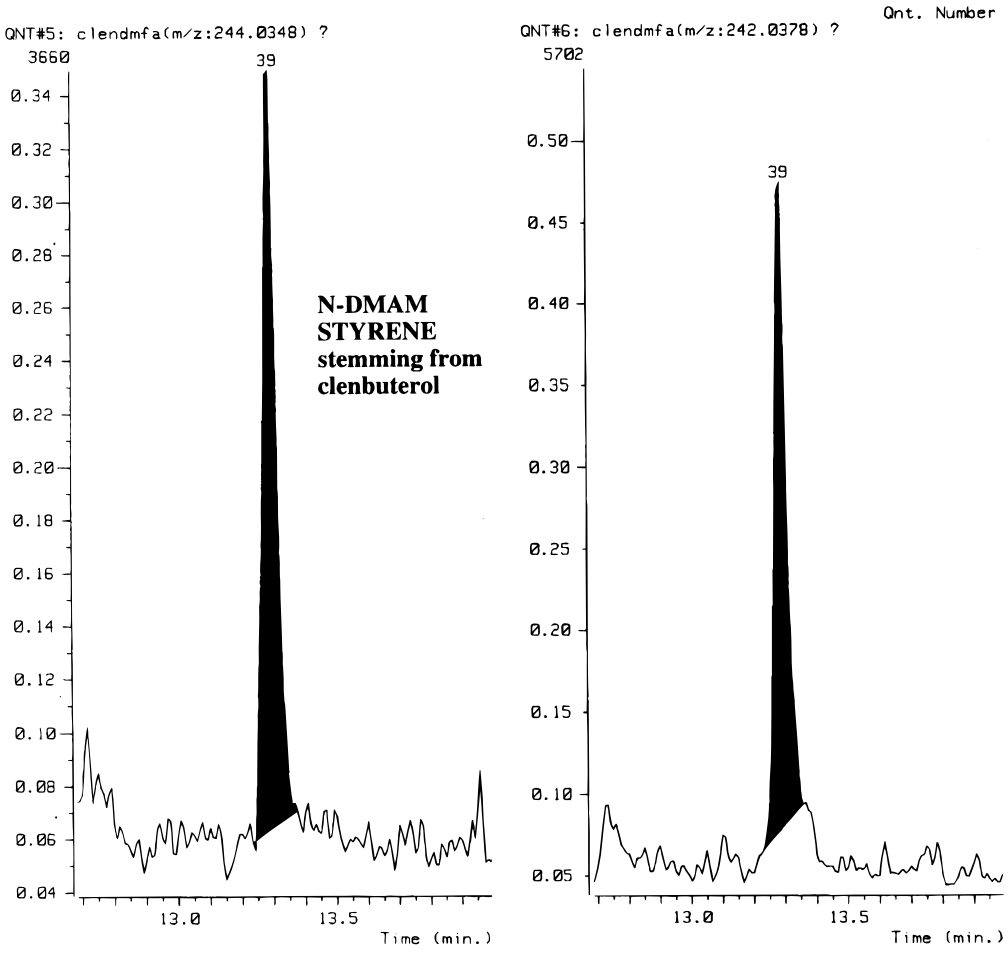


Figure 17. Calf urine sample containing 40 pg ml^{-1} of clenbuterol (N-DMAM-styrene, GC/EI-HRMS (SIM mode), R ≈ 10 000, injection in the splitless mode at 280 °C) : chromatographic profiles obtained at m/z 242.0378 and 244.0348.

and devoted to the screening as well as the confirmation steps. Actually, styrene and azetidine derivatives exhibit EI mass spectra containing a large number of abundant ions which can be selected for diagnostic purposes. Moreover, this technique would appear to be extremely helpful when one attempts to elucidate the structure of 'new' illegally used β-agonists, which has already been proved to be difficult with conventional analytical tools and usually requires the combination of several derivatization and analytical techniques.[41]

Mechanisms observed for salbutamol can also be considered as an innovative result. Carboxylic acids as well as phenol and thiol functions have actually been reported to react with DMF-DMA to give the corresponding alkylated derivatives.[42] Nevertheless, this phenomenon has not been observed for the phenol-containing β-agonists which were studied here, since O-TBDMS-styrenes predominate under the above-mentioned operating conditions. Apart from cis-diol group acetalization,[42] free hydroxy groups are known not to react with DMF-DMA, even when high temperatures are used. However, particular mechanisms were pointed out for salbutamol since DMF-DMA reacts with its primary alcohol to give a dimethylaminomethyl instead of a methoxymethyl substituent. Several MS techniques were used in order to understand these phenomena and to identify polar and thermosensitive compounds. FAB ionization is classically recognized to be useful for the analysis of high molecular mass and mildly or non-volatile molecules. However, the interest of such a MS technique for the identification of low molecular mass compounds has been demonstrated in the present work. These last findings were also pointed out recently for aziridines and their 2-chloroethylamine precursors[43] and acetylcholine.[44]

The DMF-DMA technique has also been used for metoprolol and might therefore be useful for the analysis of β-blocking agents. Further, the ability of DMF-DMA to derivatize primary alcohols to give dimethylamino groups has been investigated for the corticosteroid dexamethasone. Preliminary results did not show at first the expected transformation of the primary alcohol into a dimethylamino group as occurs for salbutamol,[45] but further investigations on reaction mechanisms have revealed that this component was effectively obtained for dexamethasone but remained unstable owing to the α-keto group. Further research is being carried out to extend the studied mechanisms to other secondary ethanolamines and other alcohol-containing compounds.

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