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Review

Bioanalysis of β_2-agonists by hyphenated chromatographic and mass spectrometric techniques

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Abstract

Recent reports on the misuse of β_2-agonists, both as stimulants and as "anabolic agents" in sports, highlight the importance of screening and confirmation methods for these compounds in anti-doping control procedures. Although only a few analytical methods have been developed for this purpose, the large experience gained, both in pharmacokinetic studies and above all in the control of the residues of β_2-agonists in animal fluids and tissues, can be of great help in the anti-doping field. This paper reviews single-residue (SR) and multi-residue (MR) methods developed for the analysis of β_2-agonists in urine, plasma and hair samples, based on hyphenated chromatographic and mass spectrometric techniques, published in the last ten-year period. The evolution from GC–MS analysis after derivatization, with particular attention to the features of different proposed derivatives, to the most recent applications of coupled-column liquid chromatography (LC–LC) combined with tandem mass spectrometric detection (MS–MS) via a thermospray (TSP) interface is illustrated, and future perspectives in the field are outlined.

Keywords: β_2-Agonists; Doping

Contents

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1. Introduction

Adrenergic β_2-agonists are a relatively recent group of drugs, originally designed for the treatment of pulmonary diseases. Together with the main pharmacological action of broncodilation, β_2-agonists produce, at higher doses, side effects on protein synthesis and lipolysis, resulting in an "anabolic" action [1].

There are, therefore, two different reasons accounting for the high potential of misuse of β_2-agonists by athletes:

(a) The stimulatory activity on respiration and on the central nervous system make these compounds attractive for their possible effects on racing performance. Hence, the use of β_2-agonists has been subjected to strict limitations, or, in the case of some compounds (i.e. clenbuterol), has been banned by the Medical Commission of the International Olympic Committee [2].

(b) In the middle of the 1980s it was demonstrated that the administration of high doses of β_2-agonists to animals exerts a growth-promoting action. Furthermore, compared to anabolic steroids, β_2-agonists possess a more selective action on muscular tissue and produce an increase in the lean meat-to-fat ratio. Although banned, the practice of administering β_2-agonists in the diet of animals has become very common, as evidenced by numerous cases of collective human poisoning, caused by the presence of active residues of β_2-agonists in meat [3,4]. In 1991, indications of a possible use of β_2-agonists, and particularly of clenbuterol, by athletes interested more in the "anabolic" action rather than in the broncodilator effect were reported. A number of important doping cases involving clenbuterol revealed before and during the Barcelona '92 Olympic Games [5,6] gave strong evidence of this second type of misuse.

The detection of residues of β_2-agonists in biosamples represents a difficult analytical problem, due to the need to attain detection limits below the ng/ml level, and also due to the relatively high polarity of these compounds. The aim of this paper is to provide a critical review of methods applied to the bioanalysis of β_2-agonists. With few exceptions, the great majority of the methods available in the literature have not been developed for anti-doping purposes, but have been applied to pharmacokinetic studies in man and animals or to the detection of residues in animal fluids and tissues, to ascertain illicit administration for growth-promoting purposes. Although many different biological matrices have been used as substrates for the analysis of β_2-agonists, only urine, plasma and hair have been considered here for their present, or potential, application in anti-doping control.

Many different approaches to the bioanalysis of β_2-agonists have been described. However, those based on mass spectrometric detection should be preferred, due to their better legal defensibility. Apart from very few exceptions [7,8], gas [9–34] or liquid [22,35–43] chromatography is used to attain the separation of analytes before MS detection. Therefore, attention has been focused on the hyphenation of chromatography and mass spectrometry as the approach of choice for β_2-agonists.

After a brief recall of the chemical and pharmacokinetic properties of the class, with reference to the analytical implications, the evolution of the bioanalysis of β_2-agonists by hyphenated chromatography–mass spectrometry over the last ten years is illustrated.

1.1. Chemical properties of β_2-agonists

The structure of β_2-agonists is closely related to that of endogenous cathecolamines. They are, in fact, phenyl β-ethanolamines bearing different substituents at the amino nitrogen (in most cases a *tert.*-butyl or isopropyl group) as well as at different positions in the phenylic ring (such as -OH, -Cl, $-NH_2$, -CN and $-CF_3$). More than 50 β_2-agonists have been described [44] and new ones have been identified in preparations for the illicit market. Fig. 1 shows the structure of the main representatives of the class.

There are two features that mainly influence the chemical behaviour of β_2-agonists and have, therefore, important analytical consequences: (a) the presence of the amino nitrogen which accounts for the basic nature of these drugs, and (b) the β-hydroxyl group, responsible for their relatively high polarity, which is increased further when other aromatic or aliphatic hydroxyl groups are present in the molecule (e.g. salbutamol). The presence of one or more phenolic groups, moreover, modifies the charge distribution in the molecule, resulting in

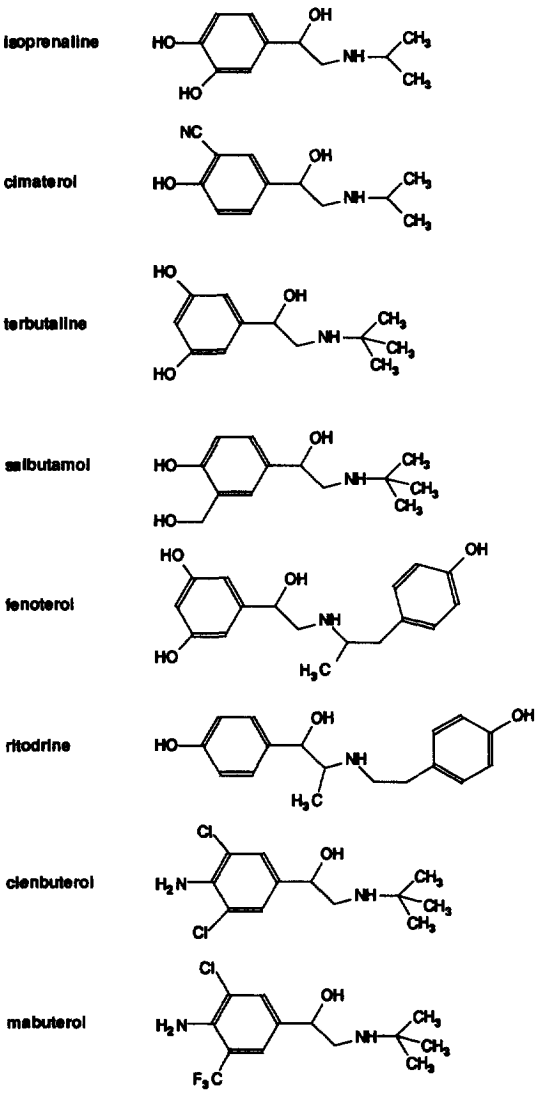


Fig. 1. Structures of different β_2-agonists.

serious implications for multi-residue (MR) extraction procedures: at a highly basic pH ($\geq$12) suitable, for example, for the extraction of clenbuterol, other β_2-agonists, such as salbutamol or terbutaline, cannot be satisfactorily extracted.

1.2. Pharmacokinetics of β_2-agonists

With regard to their pharmacokinetic features, β_2-agonists can be divided into two main groups:
(1) "low polar" β_2-agonists (e.g. clenbuterol). They are therapeutically active at very low oral doses (20–40 μg) and are characterised by peak plasma levels far below the ng/ml level after a single therapeutic dose and by relatively long half-lives (about 30 h in the case of clenbuterol). They are mainly excreted in urine as phase I metabolites, with a significant amount in the unmodified form. Urinary levels following therapeutic doses never exceed the 10–20 ng/ml level [45].
(2) "highly polar" β_2-agonists (e.g. salbutamol and ritodrine). They are characterised by notably higher therapeutical oral doses (2–10 mg), with correspondingly higher peak plasma levels (1 to 40 ng/ml). Plasma half-lives are significantly shorter (1–7 h). Urinary excretion accounts for 35 to 90% of the administered dose and phase II metabolites (glucuronides and sulphates) are prevalent [46,47].

To the author's knowledge, no data on the pharmacokinetics of β_2-agonists at high doses in man are available. Half-lives of urinary residues in the range of 1–3 days have been reported for clenbuterol and salbutamol in animals, allowing the detection of residues up to 2–3 weeks after treatment [20].

Very limited information is available on the incorporation of β_2-agonists into hair. It has been recently discovered that pigmented tissues of the eye are an accumulation site of clenbuterol and salmeterol, and that binding to melanin is involved [48]. This hypothesis may explain the observed incorporation of clenbuterol into the hair of treated animals [49]. Salbutamol, on the other hand, binds to melanin to a lesser extent [48], and its higher polarity compared to that of clenbuterol could explain its different behaviour. It is known, in fact, that low polarity drugs accumulate in hair much more than their more polar metabolites (e.g. cocaine vs. benzoylecgonine [50], heroin and 6-monoacetylmorphine vs. morphine [51]). Further research is needed in this field. Nonetheless, if these findings are confirmed, hair could become an attractive matrix to ascertain illicit use of β_2-agonists. Besides the practical and methodological advantages of hair compared to other matrices [52] — the most important being the possibility of revealing a recent historical record of drug exposure — hair analysis could in fact provide complementary information to urinalysis in anti-doping control, allowing the theoretical possibility of discriminating between acute administration, to achieve the stimulatory effect, and

chronic use, necessary to obtain the "anabolic" effect.

2. Extraction of β_2-agonists from biosamples

2.1. Urine

Isolation of the unmodified β_2-agonists from human [9,12,15,17,21,23,27,29] or animal [7,16,18–20,23–26,28,30,31,33,35,38,53] urine has been attained by liquid–liquid partition (LLE) [9,15–17,24,27,35,38], solid-phase extraction (SPE) [7,12,18,19,21,23,26,29], and immunoaffinity chromatography (IAC) [20,28,30,33,53]. Both MR methods [7,18–20,26,29,30,33,53] and single-residue (SR) methods, mainly focused on the analysis of clenbuterol [9,12,15–17,21,23,24,27,28,34,35,38,40,41], are available in the literature.

LLE from basic urine (pH 9.5 or higher) with diethyl ether [24,35], ethyl acetate [15,16] or solvent mixtures, such as *tert.*-butyl methyl ether–*n*-butanol (9:1, v/v) [17,38] and diethyl ether–2-propanol (9:1, v/v) [27] has proved to be highly efficient for clenbuterol, whereas it appears to be problematic for the more polar β_2-agonists, such as salbutamol and terbutaline. Moreover, as previously mentioned, the pH of urine may have a strong influence on the recovery of these compounds, and has to be accurately adjusted before extraction. An optimum recovery (about 70%) has been experimentally determined for terbutaline from aqueous media at pH 9.8 [9]. Either washing the sample before extraction [17] or back-extraction [9,15,16,24,35] are sometimes adopted to obtain a further clean-up.

SPE seems to provide higher versatility, as it is usually applied to MR analysis. Black and Hansson [29] have tested Sep-Pak C_{18} and XRDAH515 XTrackT columns for the extraction of six different β_2-agonists, and found the latter sorbent to produce cleaner extracts. Ayotte [21] has applied a screening method for anabolic steroids, involving SPE with Sep-Pak C_{18} columns, followed by extraction with diethyl ether, to the isolation of clenbuterol from urine, while C_2 columns have been used for clenbuterol by Schmid and Bücheler [12]. SPE using mixed-mode sorbents, e.g. containing both hydrophobic and cation-exchange functional groups, such as Bond-Elut Certify [18,19,23], Clean Screen DAU [26] or XTrackT [7], has also provided successful results. In the methods using Bond-Elut Certify columns, the buffered (pH 6) urine sample is loaded on a preconditioned column and analytes are retained on the phase by means of non-polar or weakly polar interactions. After rinsing with water, a solution of acetic acid (1 *M*) is withdrawn through the column and the interaction of basic analytes with the cation exchange groups takes place. A rinse with pure methanol can then be applied, allowing the removal of non-polar or acidic interferences and providing therefore a significant clean-up. Elution of basic analytes is then carried out with solvent mixtures, typically dichloromethane–2-propanol (4:1, v/v), containing 2–4% ammonia. A clean-up step before SPE, consisting of a wash of the acidified urine with *tert.*-butyl methyl ether, allows an improvement in the recovery of clenbuterol from bovine urine [23], while an increase from 2 to 4% of the percentage of ammonia in the final eluent improves the recovery of the more polar β_2-agonists [26].

IAC also appears to be particularly suitable for a MR approach to β_2-agonists [20,28,30,33,53]. Due to the high selectivity of the antigen–antibody reaction, a significant clean-up is achieved, and if multiple-antibody columns that allow the simultaneous trapping of β_2-agonists containing different functional groups are used, IAC may provide a broad-spectrum isolation method for this class of drugs [20].

Independently from the isolation method adopted, an enzymatic hydrolysis step is usually performed in MR methods before extraction to free the conjugated forms of β_2-agonists [7,9,20,26].

Using coupled-column liquid chromatography (LC–LC) combined with tandem mass spectrometric detection (MS–MS) via a thermospray (TSP) interface, direct injection of the filtered sample can be performed, thus eliminating the extraction step [40,41].

2.2. Plasma

LLE, using the same procedure developed for urine, has been adopted to isolate clenbuterol [15,16] and terbutaline [9] from plasma. Similarly, Förster et al. [13] have used LLE with *tert.*-butyl methyl ether,

after adjusting the pH to 9.5, followed by back-extraction with 0.2 *M* sulphuric acid. Terbutaline has been isolated from plasma also by ion-exchange extraction [10]. Terbutaline, salbutamol and fenoterol have been extracted from serum by SPE with Bond-Elut C_{18} cartridges, that were found to provide better recoveries than Sep-Pak C_{18} [11]. Elution was performed with a methanol–acetonitrile mixture (85:15, v/v), discarding the first 150–200 μl. The same compounds plus orciprenaline and fenoterol have been isolated from plasma using Sep-Pak C_{18} columns eluted with acetonitrile–5.2 m*M* ammonium acetate, pH 7.0 (77:23, v/v), obtaining recoveries of 67–104% at the 5 ng/ml level [42,43]. Direct injection of the diluted plasma sample, corresponding to 0.5 μl of plasma, has been used by Lindberg et al. [36] to determine the β_2-agonist bambuterol by LC–TSP-MS.

2.3. Hair

Although some methods have been proposed to extract clenbuterol [32,48,49] and salbutamol [32] from hair, involving methanol extraction followed by IAC [49], enzymatic digestion [48] or chemical digestion followed by SPE with Bond-Elut Certify columns [32], two of them have adopted immunochemical detection, and only one has been applied to GC–MS analysis. This involves a washing procedure with a 0.1% solution of sodium dodecyl hydrogensulphate and complete digestion of guinea pig hair (100 mg), obtained by incubating the sample with 2 ml of 2 *M* NaOH at 80°C for 30 min. Then, the pH is adjusted to 5–6 and the centrifuged hair digest is submitted to the SPE procedure with Bond-Elut Certify columns as previously described for urine [23]. Recovery was found to be higher for clenbuterol (87%) than for salbutamol (67%).

3. Instrumental analysis

As for many other classes of drugs, the evolution of hyphenated chromatographic–mass spectrometric methods devoted to the analysis of β_2-agonists has followed the progress of technology in the interfacing of chromatographic techniques to MS. Until the end of the 1980s, effective LC–MS interfaces were still not available for routine analysis, and GC–MS was the only technique at hand. Therefore, although LC has significant advantages over GC for the separation of β_2-agonists, such as the lack of requirement for derivatisation and a good solvent compatibility of urine and plasma samples with the chromatographic system, GC–MS methods were developed first. The recent output of new LC–MS interfaces, such as TSP and electrospray (ESI), however, has opened new possibilities in this field.

3.1. GC–MS

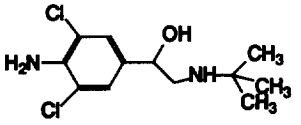
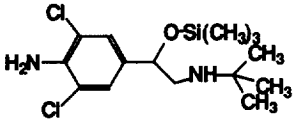
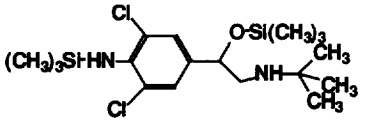
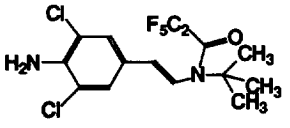
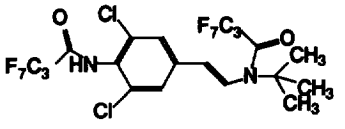
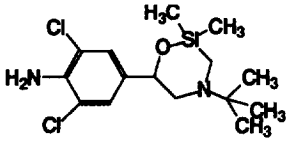
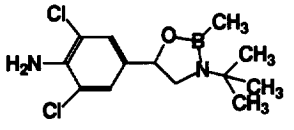
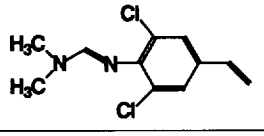
The great majority of methods applied to β_2-agonists available in the literature are based on GC–MS analysis after derivatization [9–34].

The unusually high number of different derivatives described for β_2-agonists (Table 1) emphasises how derivatization is a critical step in the GC–MS analysis of these compounds. Indeed, derivatization is needed not only to improve the poor gas chromatographic performance of β_2-agonists, but also to modify their fragmentation under electron impact (EI). Due to their β-ethanolamine structure, in fact, the EI fragmentation of β_2-agonists is dominated by the α-cleavage reaction induced by the nitrogen atom, leading to very weak or absent molecular ions, and to a base peak at low molecular mass, corresponding to the fragment $[CH_2{=}NH{-}R]^+$ (*m/z* 86 in the case of N-*tert.*-butyl β_2-agonists), with a considerable loss of mass spectral selectivity. The typical fragments of the EI spectrum of clenbuterol are listed in Table 1A, together with their relative abundances [54].

O-Silyl [9–13,18,22,26,28,33], O-acyl, N-acyl [9,15,25,34], and O-silyl, N-acyl [9] derivatives have been proposed, with some improvement in EI mass spectral selectivity for highly polar β_2-agonists containing additional hydroxyl groups in the molecule. The Tris–trimethylsilyl (TMS) derivatives of salbutamol [22] and terbutaline [9], for example, show intense fragment ions at *m/z* 369 and 356, respectively, corresponding to the protonated fragments complementary to *m/z* 86. No improvement, however, is obtained in the case of the mono-TMS derivative of clenbuterol [17] (Table 1B).

It has been shown recently that the addition of ammonium iodide [27,32] as a catalyst to the silylat-

Table 1
Structures and typical fragment ions of different derivatives of clenbuterol

	Derivative	Molecular structure	Molecular ion and typical fragments (abundance)	Reference
A	-		276 (0); 243 (3); 127 (16); 86 (100); 57 (32)	[54]
B	TMS		348 (0); 333 (1); 262 (10); 86 (100); 57 (41)	[17]
C	bis TMS		420 (0); 405 (3); 335 (41); 300 (17); 86 (100)	[21]
D	PFP		404 (8); 368 (100); 350 (14); 297 (83)[a]	[15]
E	bis HFB		650 (0); 649 (2); 573 (100); 537 (20)[b]	[34]
F	DMS		346 (20); 331 (98); 289 (47); 100 (100)	[18]
G	MBA		300 (26); 285 (64); 245 (69); 243 (100)	[23]
H	DMFDMA		242 (51); 244(31); 209 (33); 207 (100)	[31]

[a]Negative ions spectrum obtained by CI with methane.
[b]Negative ions spectrum obtained by CI with ammonia; TMS = trimethylsilyl; PFP = pentafluoropropionate; HFB = heptafluorobutyrate; DMS = dimethylsilamorpholine; MBA = methylboronate; DMFDMA = derivative with dimethylformamide dimethylacetamide.

ing agent N-methyl, N-trimethylsilyl trifluoroacetamide (MSTFA) leads to the formation of the bis-TMS derivative of clenbuterol, because of the additional silylation of the phenyl amino group (Table 1C). The resulting EI mass spectrum shows two relatively intense ions at *m/z* 335 and 300, respectively, but the molecular ion is still absent.

The low specificity of the EI mass spectra of TMS derivatives can be exploited, by converse, for screening purposes (Fig. 2). For example, by monitoring the signal at *m/z* 86, all the N-*tert.*-butyl β_2-agonists can be detected [20,26].

Chemical ionisation (CI) instead of EI may be adopted to improve selectivity. Positive ion CI with methane, isobutane or ammonia as the reactant gases has been applied to the detection of the silyl derivatives of different β_2-agonists [10–13,26,28,33], with ammonia giving the highest sensitivity. Girault et al. [15,16] have adopted negative ion CI with methane as the reactant gas to the derivative of clenbuterol obtained by reaction with pentafluoropropionic (PFP) anhydride at room temperature, obtaining a considerable gain in sensitivity that allowed the determination of the analyte at the pg/ml level. Because of the immediate loss of one PFP substituent from the O-PFP, N-PFP derivative, followed by internal dehydration, the final product of the reaction has the structure reported in Table 1D. Williams and Anderson [34] reported that by reacting clenbuterol with trifluoroacetic, PFP or with heptafluorobutyric (HFB) anhydride at room temperature, a bis-acyl derivative is formed (Table 1E

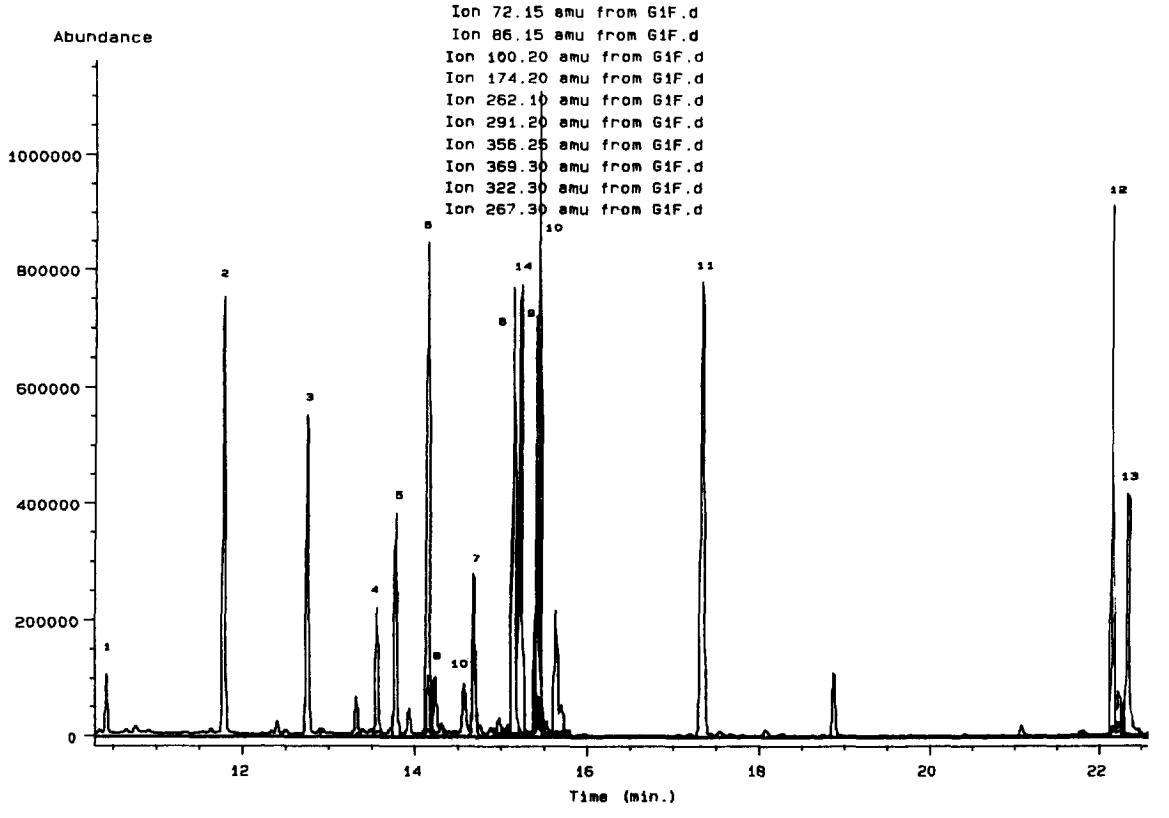


Fig. 2. EI-selected ion current profiles of a TMS-derivatized extract obtained from a blank urine sample spiked with different β_2-agonists (6–8 mg/ml). Peaks (from left to right): 1=tulobuterol·TMS (*m/z* 86); 2=mabuterol·TMS (*m/z* 86); 3=methyl-mabuterol·TMS (*m/z* 100); 4=orciprenaline·TrisTMS (*m/z* 356); 5=terbutaline·TrisTMS (*m/z* 86, 356); 6=clenbuterol·TMS (*m/z* 86, 262); 8=cimaterol·TMS (*m/z* 72); 10=methylcimaterol·TMS (*m/z* 86); 7=salbutamol·TrisTMS (*m/z* 86, 369); 8=cimaterol·bisTMS (*m/z* 72, 291); 14=metoprolol·TMS (internal standard, *m/z* 72); 9=methylclenbuterol·TMS (*m/z* 100, 262); 10=methylcimaterol·bisTMS (m/z 86, 291); 11=NA1141·bisTMS (*m/z* 174); 12=fenoterol·tetraTMS (*m/z* 322); 13=ractopamine·TrisTMS (*m/z* 267). Reproduced with permission from Ref. [26].

shows the structure of the bis-HFB derivative), due to the additional derivatization of the phenyl amino group, which is suitable for MS detection using negative ion CI with ammonia.

Another strategy that has been successfully adopted to improve mass spectral selectivity is the formation of cyclic derivatives of the bifunctional β-ethanolamine group. The cyclic dimethylsilamorpholine (DMS) derivative of clenbuterol (Table 1F), proposed by Dumasia and Houghton [18], exhibits under EI a sufficiently abundant molecular ion at m/z 346 and two characteristic ions due to the loss of $[CH_3]$ (m/z 331, base peak) and $[C(CH_3)_3]$ (m/z 289), respectively. The cyclic DMS derivative of mabuterol undergoes similar fragmentation [26].

Cyclic boronate derivatives of clenbuterol [17,23,24] and salbutamol [14,32] have been described also, with the methylboronate (MBA) derivative of clenbuterol showing much better gas chromatographic properties than the N-butyl or phenyl derivative [23]. As in the case of the cyclic DMS derivative, the mass spectrum of clenbuterol–MBA (Table 1 G) shows the most abundant ions in the high mass region: the molecular ion at m/z 300, with a characteristic isotopic pattern due to the presence of two chlorine and one boron atoms in the structure, the $[M-15]^+$ ion at m/z 285 and the ion at m/z 243, which has been demonstrated to be due to the combined loss of $[CH_3]$ and $[OBCH_3]$ from the molecular ion [23,24]. Cyclic boronate derivatives have been successfully applied to the confirmation of clenbuterol in enzyme-linked immunosorbent assay (ELISA)-positive urine samples at the Barcelona '92 Olympic Games [23], allowing the positive identification of the analyte at the ng/ml level in scan mode (Fig. 3). They have also been proved to be suitable for the determination of clenbuterol and salbutamol in the hair of treated animals, and were found to give more structural information and much less chemical noise than the corresponding TMS derivatives [32].

The main disadvantage of cyclic derivatization, however, is its unsuitability to a MR approach, as β_2-agonists containing additional hydroxy substituents may give side derivatives [26]. Salbutamol probably represents the only exception, as the relative position of the two additional hydroxyl groups in the molecule leads to the formation of the bis-MBA derivative, as shown in Fig. 4 [14,32].

An interesting method of derivatization for β_2-agonists has been recently proposed by André et al. [31]. Dimethylformamide dimethylacetamide (DMFDMA) reacts with the amino group of β_2-agonists causing the cleavage of the C–N bond and leading to the formation of a substituted styrene. In the case of clenbuterol, the phenylamino group also reacts with DMFDMA, leading to the structure shown in Table 1H. Despite the laborious derivatization (β_2-agonists containing additional hydroxyl groups require a further derivatization step, such as silylation), this method has both the advantages of providing good mass spectral selectivity, although with a reduction of molecular masses, and of being suitable to a MR approach, as the loss of the part containing the substituted amino group leads to a considerable reduction in the possible structures, originating from the different β_2-agonists, that have to be monitored by GC–MS.

No matter which derivative is formed, a good gas chromatographic separation is usually attained on fused-silica capillary columns with either methylsilicone [18,20,22,26,29–32] or 5% phenyl methyl silicone [14,17,23,27,28] stationary phases.

MS detection is always carried out by monitoring up to four selected ion fragments per analyte for levels below, or around, the ng/ml level, while full scan mode is rarely applied [23,32]. Detection limits in the range 0.1–1 ng/ml in urine and plasma and 5–10 pg/mg in hair, are reported for methods involving EI, while CI usually allows a notable improvement in sensitivity, with detection limits of 0.005 [15] and 0.015 [13] ng/ml reported for clenbuterol. Recently, the applicability of the criteria adopted by the EU for identification and quantification of residues of hormones and thyrostats (Decision 93/256/EEC) has been evaluated on different derivatives of β_2-agonists, both in the EI and CI mode [55]. According to these criteria, at least four diagnostic ions have to be monitored and the deviation of the relative abundances must be below 10%, with reference to the standard analyte. None of the derivatives, in both of the ionisation modes, fitted with these criteria, inducing the authors to suggest the enlargement of the margins for minor ions.

3.2. GC–MS–MS

MS–MS detection after GC separation has been used to detect eight different β_2-agonists as their

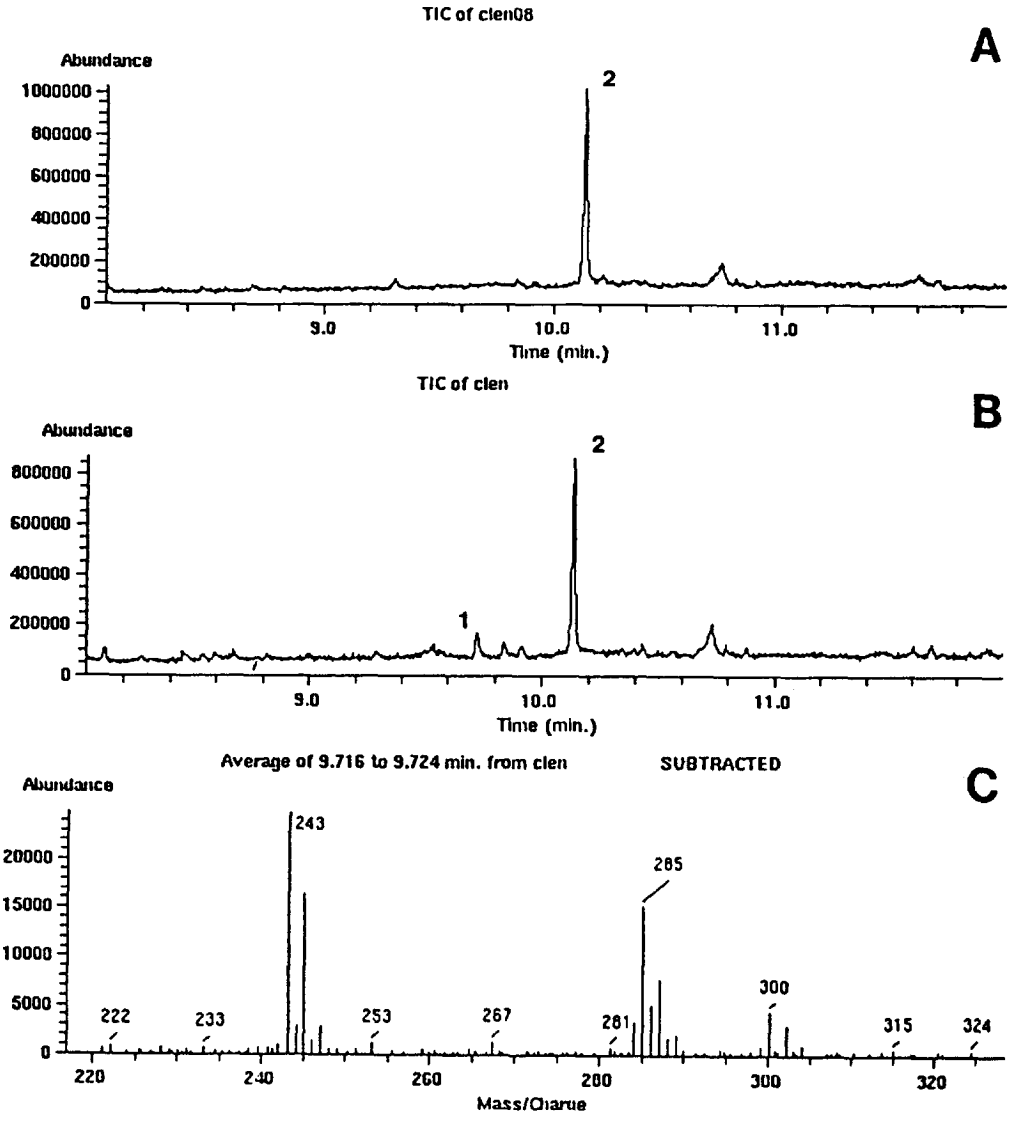


Fig. 3. GC–MS confirmation of clenbuterol (methylboronate derivative) in an ELISA-positive sample. Total ion chromatograms of blank human urine (A), ELISA-positive (~2.6 ng/ml) human urine sample (B) and the EI mass spectrum of the peak identified as clenbuterol (C). Peaks: 1=clenbuterol and 2=penbutolol (internal standard). Reproduced with permission from Ref. [23].

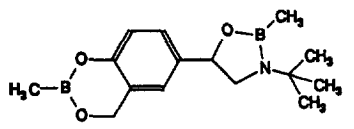


Fig. 4. Structure of the bis-MBS derivative of salbutamol.

TMS derivatives in bovine urine extracts [19]. The tandem mass spectrometer was operated in the selected reaction monitoring (SRM) mode: $[MH]^+$ ions obtained by CI with isobutane were selected as parents and submitted to collisionally induced dissociation (CID) with argon at collision energies varying from −30 to −50 eV. Two or three daughter ions were monitored for each compound. A substantial improvement in selectivity compared to conventional GC–MS was obtained in the analysis of

bovine urine and liver extracts (Fig. 5), allowing limits of detection below the 0.5 ng/ml level to be reached for most analytes.

3.3. LC–MS

The first method for the bioanalysis of β_2-agonists by LC–MS has been described by Blanchflower and Kennedy in 1989 [35] and was focused on the detection and quantification of clenbuterol residues in bovine urine. After LLE, chromatography of clenbuterol was performed by reversed-phase (RP) LC on a C_{18} column with a mobile phase consisting of 20% acetonitrile, 5% tetrahydrofuran, and 75% 0.1 *M* ammonium acetate, at a flow-rate of 1 ml/min. The column eluent was introduced into the MS

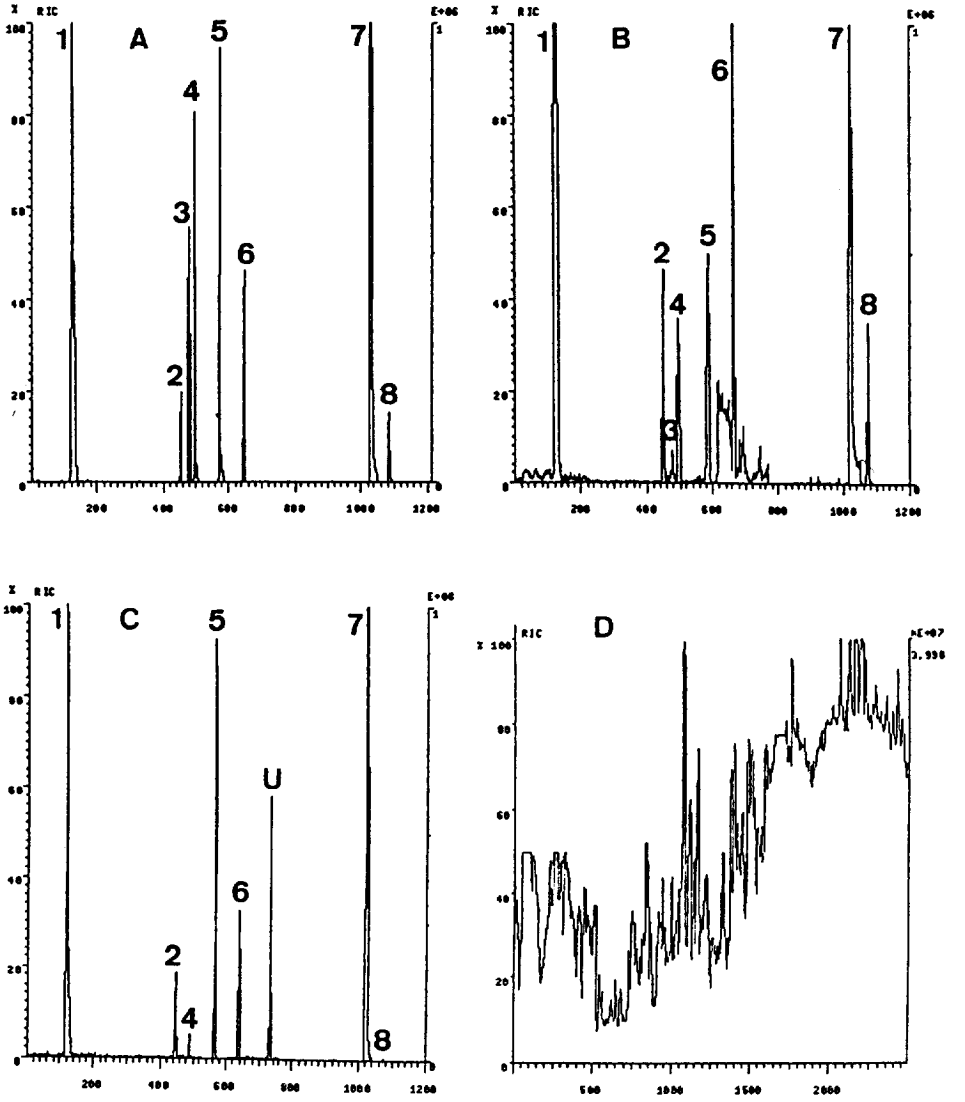


Fig. 5. Comparison of GC–MS and GC–MS–MS in the bioanalysis of the TMS derivatives of β_2-agonists. (A), (B) and (C) are standard mixture (0.2 ng), an extract from blank bovine urine spiked at 1 ppb and an extract from blank bovine liver spiked at 2 ppb, respectively, analysed by GC–MS–MS (see Section 3.2 for analytical conditions). (D) Same liver extract as (C) analysed by GC–MS in the selected ion monitoring mode. Peaks: 1=tulobuterol·TMS; 2=mabuterol·bisTMS; 3=orciprenaline·TrisTMS; 4=terbutaline·TrisTMS, 5=salbutamol·TrisTMS; 6=clenbuterol·bisTMS; ritodrine·TrisTMS; 8=fenoterol·tetraTMS; U=unknown. Reproduced with permission from Ref. [19].

via a TSP interface operated in the discharge ionisation mode. Under these conditions, clenbuterol showed little retention (3.1 min), and selectivity was attained by monitoring the $[MH]^+$ ion at m/z 277, reaching a limit of quantification of 1 ng/ml.

TSP mass spectra of β_2-agonists can be easily obtained by buffer ionisation without any additional source of energy. However, they are characterised by little or no fragmentation, almost all the signal pertaining to the $[MH]^+$ ion and, with variable intensity, to a fragment corresponding to the loss of water [22,36,37,39]. Further fragmentation can be induced by means of an electron beam (filament), a discharge, or simply by rising the repeller voltage [22], thus increasing the number of diagnostic ions.

LC–TSP–MS is also potentially suitable for MR purposes, however, the wide range of polarity of the class of β_2-agonists requires gradient elution to obtain good chromatographic separation [22,39]. This may lead to some technical difficulties, as one of the factors that strongly affects sensitivity, the temperature of vaporiser (VT) in the TSP interface, is in turn strictly related to the percentage of organic modifier (%OM) in the mobile phase [56]. The problem can be solved by programming VT to obtain its progressive decrease as the %OM increases during the chromatographic run. By using this strategy, with a linear gradient from 15 to 55% methanol in water containing 0.1 *M* ammonium acetate, 1% glacial acetic acid, and 0.2% triethylamine, at a flow-rate of 1.2 ml/min, separation of the β_2-agonists orciprenaline, terbutaline, salbutamol, ritodrine, fenoterol and clenbuterol was attained on a Zorbax TMS column in less than 10 min (Fig. 6) [39].

TSP–MS in the negative ion mode after RPLC separation on a C_{18} column has been applied to the characterisation of the sulphate ester conjugate of salbutamol in urine [57]. Authors report that chemical degradation of the conjugate occurs when ammonium acetate is present in the mobile phase (acetonitrile–water containing 50 m*M* ammonium acetate, 2:98, v/v), while ion evaporation of the molecular anions takes place in its absence, allowing unambiguous determination of the molecular mass.

The potential of ESI in the LC–MS analysis of β_2-agonists has been investigated by Debrauwer et al. [22]. However, a direct LC–MS coupling was not available in the system and off-line analysis was adopted. A significant improvement in sensitivity was observed with respect to LC–TSP–MS. Nonetheless, ammonium acetate and trifluoroacetic acid that were present in the mobile phase used for separation appeared to significantly hinder the ESI ionisation of β_2-agonists, and a further LLE from the collected HPLC fraction containing clenbuterol was necessary. ESI spectra of β_2-agonists are quite similar to TSP ones. However, a mass spectrum containing several diagnostic ions can be easily obtained with ESI by reaction of $[MH]^+$ ions with residual gas in front of the sampling orifice of the interface (skimmer), thus performing CID with a single quadrupole mass spectrometer [22]. Using the above strategy, the authors have developed a method for the analysis of clenbuterol residues in bovine urine involving LLE and RPLC on a C_{18} column, with a mobile phase consisting of a solution of ammonium acetate (50 m*M*) and trifluoroacetic acid (8 m*M*) mixed with acetonitrile (15:85, v/v), at a flow-rate of 1.2 ml/min [38].

Lindberg et al. [36] have applied LC–TSP–MS to the detection of bambuterol in dog plasma by direct injection of the diluted sample (corresponding to 0.5 μl of plasma). RPLC was performed on a C_8 column with gradient elution (from 3 to 38% methanol in 0.1 *M* ammonium acetate buffer, pH 5, at a flow-rate of 1.4 ml/min). The method proved to be highly selective, as the baseline was always free from interfering peaks. Nonetheless, the authors do not recommend direct plasma injection because of the rapid deterioration of the chromatographic column. On the contrary, they found that raw urine samples could be injected over extended periods without any deleterious effect on column performance.

LC–APCI–MS with gradient elution has been used by Doerge et al. [42,43] to develop a screening method for β_2-agonists in plasma extracts obtained by SPE. Again, separation was carried out by RPLC on a C_{18} column. By varying the voltage of the sampling cone of the interface, fragmentation could be modulated: a low voltage (15 eV) resulted in minimal fragmentation, and the transmission of the $[MH]^+$ was therefore maximised to gain sensitivity in the selected ion monitoring mode; on the other hand, higher cone voltages (up to 30 eV) were applied to improve fragmentation by in-source CID

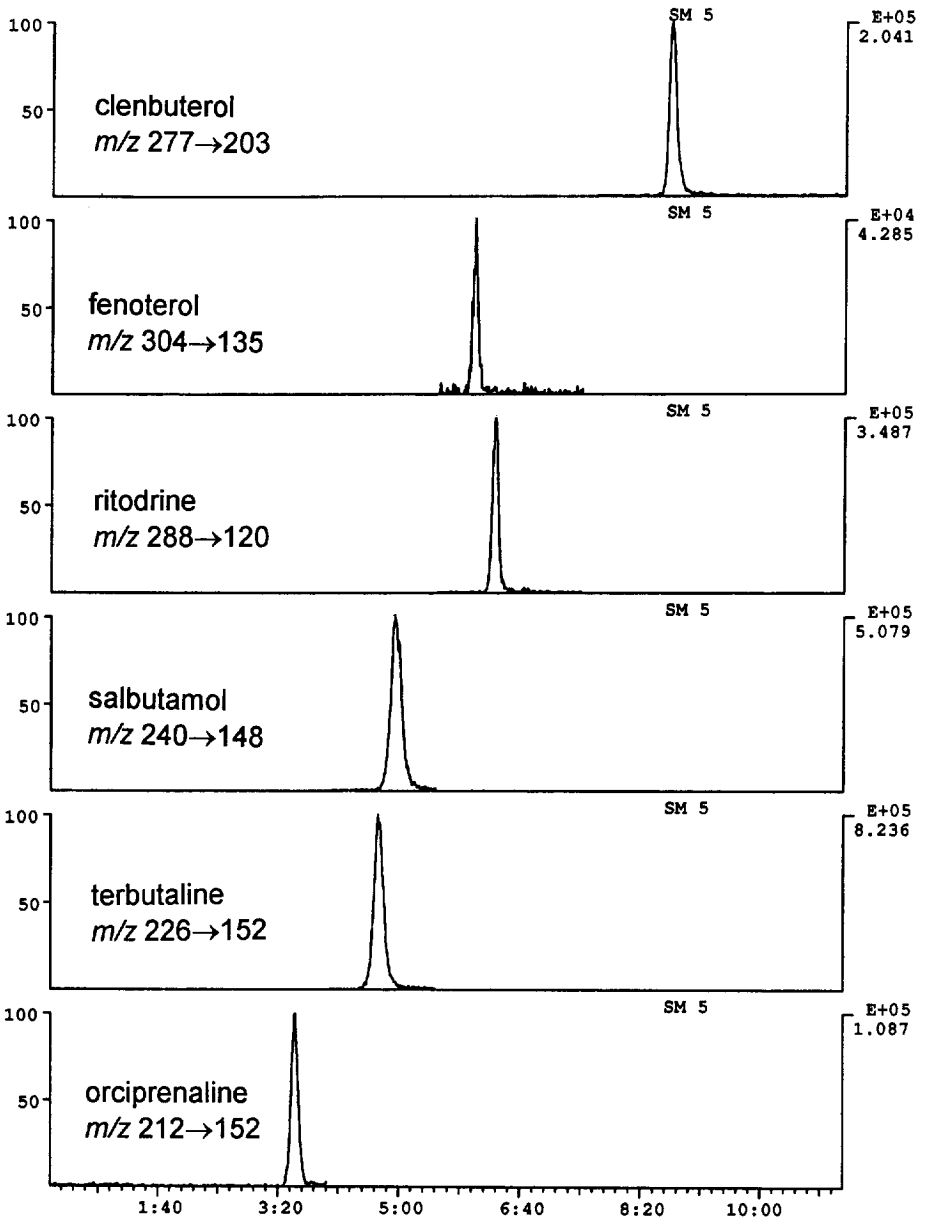


Fig. 6. LC–TSP–MS–MS analysis of six different β_2-agonists using linear gradient elution (see Section 3.3 for chromatographic conditions). In each chromatogram the analyte and the MS–MS transition monitored are indicated. Reproduced with permission from Ref. [39].

reactions, and to obtain several diagnostic fragment ions for confirmation purposes.

3.4. LC–MS–MS

Tandem mass spectrometric detection of β_2-agonists in an aqueous matrix was first used for bioanalytical purposes by van Rhijn et al. [7]. By taking advantage of the separation power of MS–MS, the authors could avoid the LC step, and highly selective detection of β_2-agonists at the ng/ml level could be achieved within a few s by flow injection analysis (FIA) via a TSP interface. Nevertheless, a sample preparation step by SPE was required, thus

reducing the actual sample throughput of the method. Extracts were injected directly into the carrier eluent (30% methanol in water containing 0.05 *M* ammonium acetate, with a flow-rate of 1.0 ml/min) and $[MH]^+$ ions were obtained by buffer ionisation. MS–MS analysis was performed in the SRM parent mode or, for screening purposes, in full scan neutral loss mode (that is, both mass analysers operating in scan mode, but with a constant mass difference). The latter mode of detection provides a powerful tool for the MR approach. The daughter ions spectra of N-*tert.*-butyl β_2-agonists obtained by collision with argon, in fact, show a characteristic peak at $[MH-74]^+$ which is due to the combined loss of water and methylpropene from the protonated molecular ion. Therefore, by using neutral loss scanning, it is possible to detect known and unknown N-*tert.*-butyl β_2-agonists contained in the sample [7].

Doerge et al. [43] have implemented the previously described LC–APCI–MS screening method in plasma by applying MS–MS detection. MS–MS was performed in the SRM mode using argon as the collision gas, at a pressure of 1.5 mtorr, and a collision energy of −15 eV. Due to the much greater signal-to-noise ratios with respect to single quadrupole MS, detection limits could be improved from 10 ng/ml to below the ng/ml level.

The possibility of direct injection of the biological sample with no or minimal sample preparation makes LC–MS an almost ideal analytical technique. However, neither LC separation followed by MS detection [35,37,42], nor FIA [7] and LC [43] with MS–MS detection allowed elimination of the sample preparation step in the analysis of β_2-agonists in urine or plasma. Recently, the potential of coupled-column liquid chromatography (LC–LC) in the bioanalysis of β_2-agonists by direct large-volume sample injection has been investigated [40]. One of the most favourable aspects of LC–LC is the utilisation of the separation power of the first column for both clean-up and sample enrichment purposes. By injecting the sample in the first column (C1) and by selecting an adequate transfer volume from the first to the second column (C2), an analytical method focused on the detection of a single analyte providing high selectivity even with traditional UV detection can be easily developed. This strategy has been successfully adopted in the set-up of a SR method for the analysis of clenbuterol residues in bovine urine [40]. By injecting 0.5 ml of filtered (0.22 μm) bovine urine in the LC–LC system (C1 and C2, C_{18} columns; mobile phase 1, 28.5% methanol in water containing 0.1 *M* ammonium acetate, 0.17 *M* formic acid, 0.01 *M* triethylamine; mobile phase 2, 35% methanol in water containing 0.1 *M* ammonium acetate and 0.01 *M* triethylamine; transfer-volume, 0.15 ml), detection of the analyte at the ng/ml level was attained. In a later work, the hyphenation of LC–LC and MS–MS via a TSP interface was applied to the direct analysis of β_2-agonists in bovine urine [41]. The study demonstrates that the hyphenation LC–LC–TSP–MS–MS is a very effective approach for the rapid, selective and sensitive analysis of β_2-agonists in urine samples (Fig. 7). LC–LC was performed using conditions similar to those described above, while MS–MS detection was attained by monitoring the reaction $[MH]^+ \rightarrow [MH-74]^+$ after collision of $[MH]^+$ with argon (collision cell pressure, 1.2–1.7 mtorr; collision energy, −15/−25 eV). With the selection of clenbuterol and salbutamol as model compounds, SR

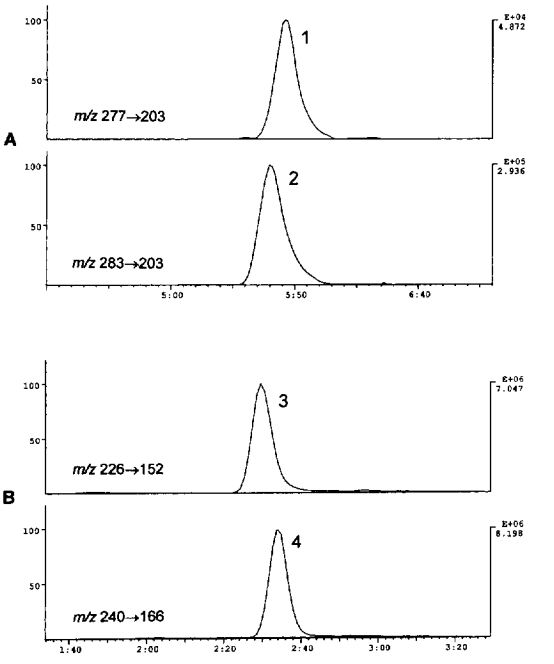


Fig. 7. Mass chromatograms obtained for the LC–LC–TSP–MS–MS analysis of clenbuterol (A) and salbutamol (B) in bovine urine by direct large volume sample injection. Peaks: 1=clenbuterol; 2=clenbuterol-d6 (internal standard); 3=terbutaline (internal standard for salbutamol); 4=salbutamol. For each analyte the MS–MS transitions are indicated.

methods were developed enabling the determination of both compounds in bovine urine at a limit of quantification of 0.1 ng/ml and with a sample throughput of 4–6 h^{-1}.

4. Conclusions

GC–MS after derivatization, although requiring laborious and time-consuming sample preparation, is still the method of choice for the bioanalysis of β_2-agonists, as proved by the much higher number of GC–MS methods published in the literature compared to LC–MS ones. This is due to both the high degree of standardisation of the technique and to the high diffusion of bench-top GC–MS systems in analytical laboratories. SPE, particularly when using mixed mode sorbents, and IAC using multiple-antibody columns, give the best results for screening purposes. The use of different derivatives for screening and for confirmation seems to be a good strategy: silylation, for example, allows a broad-spectrum screening, but does not appear to give enough EI mass spectral selectivity for confirmation purposes. On the other hand, the formation of cyclic derivatives, such as DMS and MBA ones, provides high mass spectral selectivity for some compounds (e.g. clenbuterol and mabuterol) but is not suitable for a MR approach, as highly polar compounds form side derivatives. Derivatization with DMFDMA followed by silylation of additional hydroxyl groups could be a good compromise between the two opposite demands of versatility and selectivity.

After the development of effective LC–MS interfaces, an increasing number of methods based on LC separation before MS detection have been published. Indeed, the evolution in the bioanalysis of β_2-agonists seems to follow this direction, at least for plasma and urine samples. LC, in fact, has significant advantages over GC in the separation of β_2-agonists, such as the lack of need for derivatization and the solvent compatibility of sample (urine and plasma) with the chromatographic system. The possibility of direct sample injection, in particular, appears favourable, allowing one to minimise the risk of artefact formation and to increase substantially the sample throughput by eliminating the sample preparation step. Nevertheless, interference of the matrix, particularly severe in the case of plasma, on both the chromatographic and the ionisation processes, is still a major problem. LC–LC, by utilising the separation power of the first column for both clean-up and sample enrichment purposes, has proved to be an excellent solution for the direct large-volume injection of bovine urine and, coupled with MS–MS detection, has allowed the rapid, selective and sensitive analysis of single β_2-agonists. However, its potential in the MR approach, as well as in the direct analysis of other biological matrices, such as plasma, has still to be investigated.

The analysis of sulphate and glucuronide conjugates of β_2-agonists is another almost unexplored field, although LC–TSP–MS in the negative ion mode has been successfully applied to the characterisation of the sulphate conjugate of salbutamol. Nevertheless, enzymatic hydrolysis of the sample, or even the analysis of the unconjugated quote of β_2-agonists excreted in urine, seem to provide good alternatives.

Further research to investigate the potential of hair as a target matrix for the analysis of β_2-agonists is also needed. The mechanisms of incorporation into hair have still to be fully understood, and more pharmacokinetic studies are required to know both to what extent accumulation into hair occurs, and the role played by differences in the physico-chemical behaviour of β_2-agonists. Nevertheless, hair has notable advantages over plasma and urine as its collection is harmless and can be executed without violating individual privacy. Furthermore, hair analysis gives the theoretical possibility, through the comparison with urinalysis, of a discrimination between acute administration, to achieve the stimulatory effect, from chronic use, necessary to obtain the "anabolic" effect. Although hair is not amenable to direct analysis by LC–MS, the use of this hyphenated technique for the analysis of hair digests or of hair extracts could be a convenient alternative approach to GC–MS. The applicability of direct MS–MS analysis of hair using a solids probe, successfully employed in the field of drugs of abuse [58], should be also evaluated.

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References

[1] J.P. Hanrahan, Beta-agonists and Their Effects on Animal Growth and Carcass Quality, Elsevier Applied Science, Barking, 1987.
[2] International Olympic Committee, Medical Commission, List of Doping Classes and Methods, Lausanne, CH, Letter Ref. No. LAB/ME/M/MH/C/ML/F/FR/CO/OR/33/93/rea, March 1993.
[3] J.F. Martinez-Navarro, Lancet, 336 (1991) 1311.
[4] C. Pulce, D. Lamaison, G. Keck, C. Bostvironnols, J. Nicolas and J. Descotes, Vet. Hum. Toxicol., 33 (1991) 480.
[5] Muscling in on clenbuterol (editorial), Lancet, 340 (1992) 403.
[6] Institut Municipal d'Investicaciò Medica, Internal Report of Activities, Games of the XXV Olympiad of Barcelona '92, Report IMIM/FT/DOPING/JJOO/1/1.2, Barcelona (1992).
[7] J.A. van Rhijn, M. O'Keeffe, H.H. Heskamp and S. Collins, J. Chromatogr. A, 712 (1995) 67.
[8] M.H. Lamoree, N.J. Reinhoud, U.R. Tjaden, W.M.A. Niessen and J. van der Greef, Biol. Mass Spetrom., 23 (1994) 339.
[9] R.A. Clare, D.S. Davies and T.A. Baillie, Biomed. Environ. Mass Spectrom., 6 (1979) 31.
[10] S.E. Jacobsson, S. Jönsson, C. Lindberg and L.Å. Svensson, Biomed. Environ. Mass Spectrom., 7 (1980) 265.
[11] J.G. Leferink, J. Damkers and R.A.A. Maes, J. Chromatogr., 229 (1982) 217.
[12] J. Schmid and A. Bücheler, Biomed. Environ. Mass Spectrom., 17 (1988) 415.
[13] H.J. Förster, K.L. Rominger, E. Ecker, H. Peil and A. Wittrock, Biomed. Environ. Mass Spectrom., 17 (1988) 417.
[14] J. Zamecnik, J. Anal. Toxicol., 14 (1990) 132.
[15] J. Girault, P. Gobin and J.B. Fourtillan, Biomed. Environ. Mass Spectrom., 19 (1990) 80.
[16] J. Girault and J.B. Fourtillan, J. Chromatogr., 518 (1990) 41.
[17] A. Polettini, M.C. Ricossa, A. Groppi and M. Montagna, J. Chromatogr., 564 (1991) 529.
[18] M.C. Dumasia and E. Houghton, J. Chromatogr., 564 (1991) 503.
[19] L. Leyssens, C. Driessen, A. Jacobs, J. Czech and J. Raus, J. Chromatogr., 564 (1991) 515.
[20] L.A. van Ginkel, R.W. Stephany and H.J. van Rossum, J. Assoc. Off. Anal. Chem. Int., 75 (1992) 554.
[21] C. Ayotte, Proceedings of the 10th Cologne Workshop on Dope Analysis, 7–12 June 1992, Sport und Buch Strauss Sport Edition, Cologne, 1992, pp. 185–196.
[22] L. Debrauwer, G. Delous and G. Bories, Chromatographia, 36 (1993) 218.
[23] A. Polettini, A. Groppi, M.C. Ricossa and M. Montagna, Biol. Mass Spectrom., 22 (1993) 457.
[24] W.J. Blanchflower, S.A. Hewitt, A. Cannavan, C.T. Elliott and D. Glenn, Biol. Mass Spectrom., 22 (1993) 326.
[25] R. Cozzani, B. Neri, U. Agrimi, E. Pierdominici, L. Achene and G. Brambilla, Proceedings of the 3rd International Congress on Feedborne Infection and Intoxications, Vol. 2, Berlin, 16–19 June, 1992, p. 1279.
[26] M.-P. Montrade, B. Le Bizec, F. Monteau, B. Siliart and F. André, Anal. Chim. Acta, 275 (1993) 253.
[27] Y. Xu, L. Shen, X. Liu and C. Zhang, Proceedings of the 11th Cologne Workshop on Dope Analysis, 7–12th March, 1993, Sport und Buch Strauss Sport Edition, Cologne, 1993, pp. 191–195.
[28] J.A. van Rhijn, W.A. Traag and H.H. Heskamp, J. Chromatogr., 619 (1993) 243.
[29] S.B. Black and R.C. Hansson, Presented at the 31st Meeting of T.I.A.F.T., Leipzig, August 1993.
[30] H. Hooijerink, R. Schilt, E.O. van Bennekom and F.A. Huf, J. Chromatogr. B, 660 (1994) 303.
[31] F. André, B. Le Bizec, M.P. Montrade, D. Maume, F. Monteau and P. Marchand, Analyst, 119 (1994) 2529.
[32] A. Polettini, M. Montagna, J. Segura and X. de la Torre, J. Mass Spectrom., 31 (1995) 47.
[33] J.A. van Rhijn, H.H. Heskamp, M.L. Essers, H.J. van de Watering, H.C.H. Kleijnen and A.H. Roos, J. Chromatogr. B, 665 (1995) 395.
[34] K.R. Williams and R.A. Anderson, in B. Jacob and W. Bonte (Editors), Advances in Forensic Sciences, Proceedings of the 13th Meeting of the International Association of Forensic Sciences, Vol. 5, Düsseldorf, August 22–28th, 1993, Koster, Berlin, 1995, p.124.
[35] W.J. Blanchflower and D.G. Kennedy, Biomed. Environ. Mass Spectrom., 18 (1989) 935.
[36] C. Lindberg, J. Paulson and A. Blomqvist, J. Chromatogr., 554 (1991) 215.
[37] M. Montana, G. Pompa, F. Manca, A. Marchisio, P. Gulden and G. Brambilla, Proceedings of the 3rd International Congress on Feedborne Infection and Intoxications, Vol. 2, Berlin, 16–19th June, 1992, p. 1284.
[38] L. Debrauwer and G. Bories, Anal. Chim. Acta, 275 (1993) 231.
[39] A.Polettini, in M. Montagna, O. Crippa and P. Maternini (Editors), Doping e Scienze Medico-Forensi, Proceedings of VI Convegno Nazionale Gruppo Tossicologi Forensi S.I.M.L.A., Pavia, September 1993, EDIMES, Pavia, 1993, p. 226.
[40] A. Polettini, M. Montagna, E.A. Hogendoorn, E. Dijkman, P. van Zoonen and L.A. van Ginkel, J. Chromatogr. A, 695 (1995) 19.
[41] E.A. Hogendoorn, P. van Zoonen, A. Polettini and M. Montagna, J. Mass Spectrom., (1996) in press.
[42] D.R. Doerge, S. Bajic and S. Lowes, Rapid Commun. Mass Spectrom., 7 (1993) 462.
[43] D.R. Doerge, S. Bajic, L.R. Blamkenship, S.W. Preece and M.I. Churchwell, J. Mass Spectrom., 30 (1995) 911.
[44] C.J.W. Brooks and L. Hanaineh, Biomed. J., 87 (1963) 151.
[45] A. Zimmer, Arzneim-Forsch. (Drug Res.), 26 (1976) 1446.
[46] M.E. Evans, S.R. Walker, R.T. Brittain and J.W. Paterson, Xenobiotica, 3 (1973) 113.
[47] W. Finkelstein, Drug. Intell. Clin. Pharm., 15 (1981) 425.

[48] M.J. Sauer and S.P.L. Anderson, Analyst, 119 (1994) 2553.
[49] A. Adam, N. Gervais, A. Panoyan, H. Ong, L. Beliveau, C. Ayotte and P. Delahaut, Analyst, 119 (1994) 2663.
[50] E.J. Cone, D. Yousefnejad, W.D. Darwin and T. Maguire, J. Anal. Toxicol., 15 (1991) 250.
[51] B.A. Goldberger, Y.H. Caplan, T. Maguire and E.J. Cone, J. Anal. Toxicol., 15 (1991) 226.
[52] M.R. Harkey and G.L. Henderson, in R.C. Baselt (Editor), Advances in Analytical Toxicology, Vol. 2, Year Book Medical Publishers Inc., Chicago, 1989, p. 298.
[53] L.A. van Ginkel, H.J. van Rossum and R.W. Stephany (Editors), Proceedings of the EC-Workshop: The Use of Immunoaffinity Chromatography in Multi-residue and Confirmation Analysis of β_2-agonists in Biological Samples, Bilthoven, April 1991, RIVM, Bilthoven, Netherlands, 1991.
[54] K. Pfleger, H.H. Maurer and A. Weber. Mass Spectral and GC Data of Drugs, Poisons, and Their Metabolites, VCH Verlagsgesellschaft, Weinheim, Germany, 1992.
[55] A. Preiss and B. Jülicher, Presented at the 2nd International Symposium on Hormone and Veterinary Drugs Residue Analysis, Bruges, May–June 1994.
[56] C.E.M. Heremans, R.A.M. van der Hoeven, W.M.A. Niessen, U.R. Tjaden and J. van der Greef., J. Chromatogr., 474 (1989) 149.
[57] G.D. Bowers, D.M. Highton, G.R. Manchee, J. Oxford and D.A. Saynor, J. Chromatogr., 554 (1991) 175.
[58] D.A. Kidwell, J. Forensic Sci., 38 (1993) 272.