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Confirmation Analysis of Clenbuterol in Beef Liver and Minced Beef by a Combination of Immunoaffinity Chromatography and Liquid Chromatography/Electrospray Mass Spectrometry or Liquid Chromatography/Electrospray Tandem Mass Spectrometry

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Confirmation methods based on the combination of immunoaffinity chromatography for sample clean-up and on-line liquid chromatography/mass spectrometry with electrospray ionization in both selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes were developed for the determination of clenbuterol in beef liver and meat samples. The absolute minimum detection limits are 25 pg per injection (equivalent to 0.18 ng g^{-1} of sample) in the SIM mode (using a single mass analyzer) and as low as 3–5 pg per injection (equivalent to 0.02–0.04 ng g^{-1} of sample) in the MRM mode (tandem mass spectrometry). Both mass spectrometric methods are compared and the results demonstrate that tandem mass spectrometry offers a much lower detection limit and higher specificity. A beef liver sample and a minced beef sample, both spiked at 1 and 5 ppb levels, were used as examples to demonstrate the excellent sensitivity and specificity of the proposed methods for the unequivocal quantitative determination of clenbuterol in meat products.

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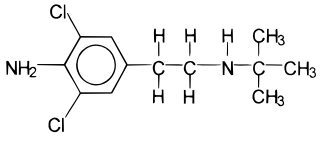
INTRODUCTION

Clenbuterol is a well known β-agonistic drug. In addition to its clinical and therapeutic values, its side-effects of enhancing muscle growth and decreasing fat deposition[1] have a significant economic benefit in commercial meat production. This has led to the illegal use of this drug and other β-agonists (such as fenoterol, metaproterenol, salbutamol and terbutaline) as growth promoters in livestock animals. Because of its acute toxicity (cardiac, central nervous system, pulmonary) in humans,[2] β-agonists have been banned in many countries. For reasons of public health, government agencies are required to ensure that edible meat products for human consumption are free from residues of β-agonists. Sensitive and specific methods are therefore required to monitor these drugs at μg kg^{-1} (ppb) levels in biological tissues or fluids.



Clenbuterol
$C_{12}H_{18}ON_2Cl_2$ (M.W. 276.08)

An effective sample clean-up procedure constitutes a crucial part in the development of good analytical methods. Immunoaffinity chromatography (IAC) has been proved recently to be a most valuable tool in the isolation and purification of samples for residue analyses.[3–5] It is based on the highly specific binding between analyte(s) and the antibody covalently attached to a solid support such as Sepharose or silica gel in the column. The target compound and its analogs can be selectively isolated from complex biological matrices by binding to the antibodies of the column and then released later with eluting solvent(s). The eluate is normally very 'clean' and free from interferences, and is thus highly suitable for quantification by subsequent instrumental analysis.

Quantitative analysis of clenbuterol is often performed by using liquid chromatography (LC) with UV detection or gas chromatography (GC) after chemical derivatization. However, both methods suffer from low sensitivity and specificity and are considered to be inadequate to measure low ppb levels of clenbuterol in

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complex biological samples. Mass spectrometry has been recognized as the most powerful tool in trace to ultra-trace analyses. Using the trimethylsilyl (TMS) ether derivative, gas chromatography/mass spectrometry (GC/MS) in the electron impact (EI) mode[6–8] and chemical ionization (CI) mode[9] were used for clenbuterol determination. Owing to excessive EI fragmentation of the TMS derivative of clenbuterol, only non-specific low-mass fragments were used, thus limiting the usefulness of this technique. GC/MS based on negative-ion chemical ionization (NICI)[10] and the pentafluoroacetyl derivative offers high sensitivity (low pg levels) but still requires chemical derivatization which may introduce unwanted variability or sample loss and lengthen the analytical procedures. Most analysts prefer to work with the analyte in its native form. In most cases, liquid chromatography/mass spectrometry (LC/MS) is the method of choice. Several LC/MS procedures using different ionization techniques have been proposed. Some are off-line[11] (LC and MS were not coupled together) or used flow injection of the sample without LC separation.[12] Doerge and co-workers[13,14] successfully used combined LC and atmospheric pressure chemical ionization (APcI) MS in the determination of clenbuterol and other β-agonists in human plasma at low ng ml^{-1} (ppb) levels. Recently, Hogendoorn et al.[15] proposed coupled-column (LC/LC) and thermospray (TSP) tandem mass spectrometry (MS/MS) for the determination of clenbuterol and salbutamol in bovine urine.

The objective of the present study was to develop an unequivocal confirmation method to identify clenbuterol by using immunoaffinity chromatography as a specific clean-up method and LC/electrospray ionization (ES) MS/MS as a highly sensitive and specific detection procedure.

EXPERIMENTAL

Reagents

Clenbuterol hydrochloride was obtained as an analytical standard from Sigma Chemical (St Louis, MO, USA). Phosphate-buffered saline (PBS) solution was prepared by dissolving 2.68 g of sodium phosphate and 8.76 g of sodium chloride in 1 l of water. The pH was adjusted to 7.4 with 0.1 M phosphoric acid.

Sample extraction

The detailed procedure for the sample extraction and clean-up was published elsewhere.[16] In general, a 12 g homogenized beef liver sample was divided into two equal portions and both were extracted and cleaned up in the same way. The first half (6 g) served as a blank while aliquots of the other half were spiked with 1 or 5 ppb of clenbuterol after extraction and sample clean-up. Each homogenate was mixed with 30 ml of aqueous 0.01 M HCl in an ultrasonic water-bath for 15 min. The mixture was heated at 80 °C for 30 min, cooled in a freezer for 10 min and then centrifuged at 16 000 rpm for 20 min at 5 °C. The supernatant was transferred into a clean flask and the pH was adjusted to 6 with 1 M NaOH.

Sample clean-up by cation-exchange chromatography and immunoaffinity chromatography

The sample extract was applied to a weak cation-exchange solid-phase extraction (SPE-COOH) cartridge (3 ml volume, 500 mg of adsorbent; Baker) previously conditioned sequentially with 10 ml of methanol, 3 ml of water, 3 ml of 0.1 M sodium monobasic phosphate buffer (pH 6) and 3 ml of water. After the sample application, the cartridge was rinsed with 4 ml of water followed by 4 ml of ethanol (the rinses were discarded). The analyte was eluted with 5 ml of 2% (v/v) ammonia solution in ethanol and the resulting effluent containing the clenbuterol was collected and evaporated under nitrogen at 30 °C to a volume of 0.1 ml. The solution was then diluted to 1 ml with PBS (pH 7.4) and applied to an immunoaffinity (IA) cartridge (RIDA Clenbuterol column, R-Biopharm, Germany) pre-washed with 2 ml of PBS (pH 7.4). The IA cartridge was rinsed with another 0.5 ml of PBS (pH 7.4) and 1 ml of 20% (v/v) ethanol in water. The clenbuterol was eluted from the cartridge with 2 ml of 80% (v/v) ethanol in water and collected in a 5 ml centrifuge tube. The eluate was evaporated to 0.4 ml and subjected to LC/MS or LC/MS/MS analysis. The recoveries of clenbuterol through the complete analytical procedure for samples spiked at 2 and 5 ng g^{-1} ranged from 76 to 105% (mean 90%) compared with pure standards carried through the same procedure. Absolute recoveries of pure clenbuterol through the same analytical steps was 70 $\pm$ 5% (n = 6). The used IA cartridge was regenerated by rinsing with 5 ml of water followed by 5 ml of PBS (pH 7.4) and stored at 4 °C until the next use.

LC conditions

A 4.5 mm i.d. $\times$ 10 cm TSK-GEL Super-ODS column (2 μm particle size, from TosoHaas, Montgomeryville, PA, USA) was used. Mobile phase A (0.15% (v/v) formic acid in 14.7 mM aqueous ammonium acetate, pH $\approx$ 3.1) and mobile phase B (0.16% (v/v) formic acid + 1.48% 14.7 mM ammonium acetate + 49.18% acetonitrile + 49.18% water) were delivered by a Hewlett-Packard HP-1100 HPLC system (including binary bumps, degasser, autosampler and variable-wavelength UV detector). The following solvent gradient program was used : the initial concentration of mobile phase B was 20%, increased to 40% at 2 min and then to 80% at 10 min and was held for an additional 2 min before it was returned to 20%. The LC effluent was also monitored with a UV detector (at 245 nm) placed in series between the LC column and the ES probe of the mass spectrometer. The total flow rate of the LC effluent at 0.6 ml min^{-1} was reduced to $\sim$ 0.1–0.2 ml min^{-1} to the mass spectrometer by means of a splitter (constructed from a low dead volume tee and the splitting ratio was adjusted by using PEEK (polyether ether ketone) tubing of different length and different internal diameter). Since electrospray ioniza-

tion is a concentration-dependent technique, the actual splitting ratio (as long as the optimum spray is maintained) does not affect the quantitative analysis.

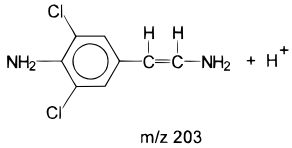
MS conditions

A Quattro II (Micromass, Altrincham, UK) tandem mass spectrometer equipped with an ES ion source was used. Microsoft Windows-NT based MassLynx software (Micromass) was used to control the instrument and for data acquisition/processing. The operating parameters were as follows: source temperature, 100 °C; nebulizing gas, 20 l h^{-1}; drying gas, 450 l h^{-1}; ES capillary voltage, 4.0 kV; cone voltage, 35 V for selected ion monitoring (SIM) and 25 V for multiple reaction monitoring (MRM); and collision gas (argon) pressure, 2×10^{-3} mbar. The skimmer lens (between the sample cone and the skimmer) voltage was set at 5 V above the cone voltage whereas the skimmer was maintained at 1.5 V. In the SIM mode, the following ions were monitored, each at a dwell time of 0.2 s: m/z 203, 205, 259, 261, 277 and 279. The ion energies for both quadrupole mass analyzers were 0.2 V. In the MRM mode, the transmission of the protonated molecular ion ($[M + H]^+$ at m/z 277) plus two fragment ion transitions (m/z 277 to 259 and m/z 277 to 203) were monitored with the settings of dwell time = 0.2 s, span = 0.1 Da and inter-channel delay = 0.02 s for each parent/fragment ion pair. Within each mass switching cycle, collision energies were programmed through the MassLynx software for each ion pair as follows : 5 eV for m/z 277 → 277; 11 eV for m/z 277 → 259 and 17 eV for m/z 277 → 203. While the parent ion resolution was set at ~10% valley separation, the baseline unit resolution was maintained for all fragment ions. SIM and MRM data were smoothed twice for every two adjacent data points to improve the peak detection.

RESULTS AND DISCUSSION

Selective ion monitoring method

Under normal ES conditions, clenbuterol produces predominantly protonated molecular ions (m/z 277) with little fragmentation. The degree of fragmentation produced by collision-induced dissociation (CID) in the ion source region can be controlled by varying the sampling cone voltage in the ES ion source. Figure 1 shows the ES mass spectra of clenbuterol at different cone voltages. Virtually, only the $[M + H]^+$ ion was observed at the cone voltage of 20 V. The dehydrated ion $[M + H - H_2O]^+$ at m/z 259 began to appear as the cone voltage increased and the m/z 203 $[M + H - H_2O - C_4H_8]^+$ ion became the most dominant peak at high cone voltage (>40 V). A similar effect was observed by Debrauwer and Bories[11] in the ES mode and Doerge et al.[13] in the APcI mode, although the reported cone voltages that produced the same fragmentation were different.



m/z 203

For SIM, it is preferred to include some diagnostic fragment ions in addition to the $[M + H]^+$ ion in order to enhance the specificity. The cone voltage at 35 V was chosen as it would produce almost equal abundances for the three major ions ($[M + H]^+$, $[M + H - H_2O]^+$ and $[M + H - H_2O - C_4H_8]^+$). Six masses (m/z 277, 279; m/z 259, 261 and m/z 203, 205) from these three ions and their ^{37}Cl isotopic peaks were monitored in the SIM mode. Figure 2 shows the SIM mass chromatograms from an injection of 100 pg of clenbuterol standard. The absolute minimum detection limit (MDL) was estimated to be around 50 pg for all six channels with signal-to-noise ratios better than 3:1. If only the ^{35}Cl peaks (i.e. m/z 277; 259 and 203) are considered for positive identification, the MDL will be reduced to around 25 pg. For a 6 g sample with a final volume of 0.42 ml and a 10 µl injection, these MDLs will be equivalent to 0.35 and 0.175 ppb, respectively, in meat. If necessary, these relative MDLs can be further improved by increasing the injection volume and/or reducing the final volume. Since m/z 277, 259 and 203 ions contain two chlorine atoms, the relative peak area ratios between 277/279; 259/261 and 203/205 should be close to the theoretical value of 100:65 and form the basis for further confirmation. When samples are run under the same conditions as standards, an additional confirmation criterion can include the relative area ratios among

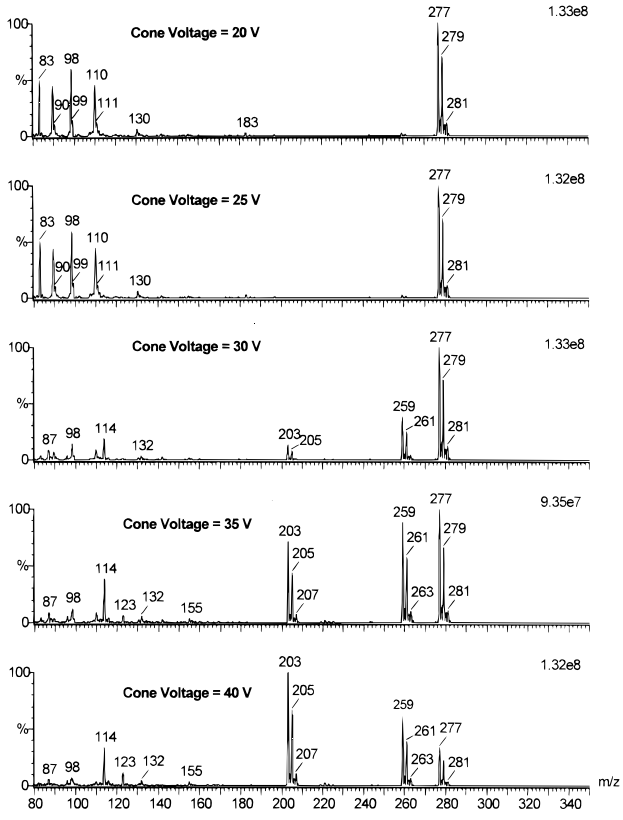


Figure 1. Electrospray mass spectra of clenbuterol at different sampling cone voltages in the ion source region. Spectra were obtained by flow injections of 5 ng of clenbuterol standard. Low-mass ions at low cone voltages could arise from solvent clusters and were not related to the parent compound.

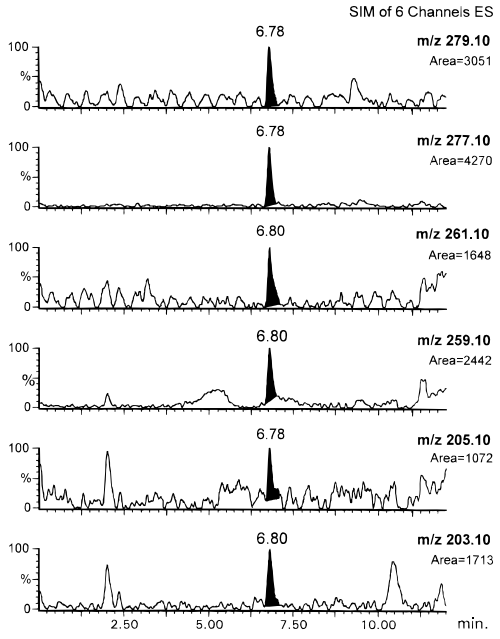


Figure 2. LC/ESMS/SIM mass chromatograms from an injection of 100 pg of clenbuterol standard. Protonated molecular ion at m/z 277 and two major fragments at m/z 259 and 203 along with their ^{37}Cl isotope ions were monitored. On-line LC was performed on a 4.5 mm i.d. × 10 cm TSK-GEL Super-ODS (2 μm particle size) column at a flow rate of 0.6 ml min^{-1}. Between 20 and 30% of the LC effluent was directed to the mass spectrometer by means of a splitter. Detailed LC conditions are described in the Experimental section.

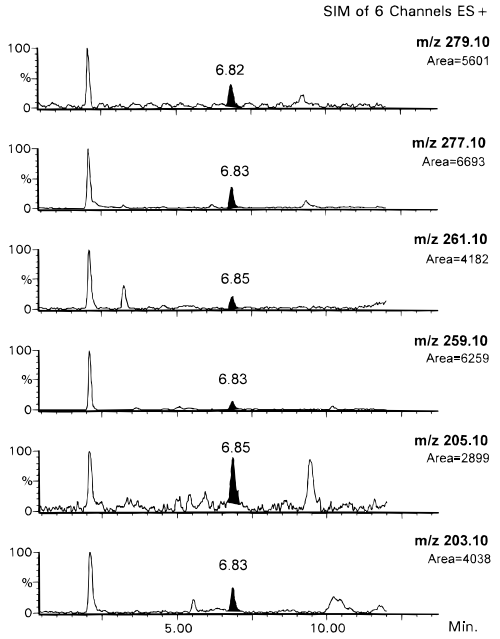


Figure 3. LC/ESMS/SIM mass chromatograms of a reagent blank processed through a regenerated IA cartridge. Positive peaks were detected at the correct retention time of clenbuterol. Data were acquired in the early stage of method development, hence the mass spectrometric conditions were slighted different from those in Fig. 2. Mass chromatograms were normalized to the largest peak in each mass channel.

these three ions (m/z 277, 259 and 203). The area responses from 100 pg to 10 ng of clenbuterol were linear ($r = 0.9990$ for m/z 277, 0.9986 for m/z 259 and 0.9987 for m/z 203). This is contrary to the variation of signals (for same three ions) observed by Debrauwer and Bories[11] when flow injection was used. Presumably, solvent impurities and other interfering compounds might have effects on their quantification. This demonstrates the benefits of direct coupling of LC and MS, which not only provides retention time information for identification but also removes interferences from the target compound to allow lower detection limits.

For economic reasons, our LC/UV screening method[16] includes a procedure to regenerate IA cartridges by washing with solvents after sample processing. In our earlier experiments, when 'blank' beef liver samples processed by regenerated IA cartridges were analyzed, positive peaks were observed in all six mass chromatograms at the correct retention time of clenbuterol, indicating the presence of clenbuterol at 0.6 ppb (determined by both the external standard method and the standard addition method as well as by the MS/MS method described in the next section; data not shown) in the sample. It was confirmed later that the contamination did not originate from the beef liver sample but from the reused IA cartridge (Fig. 3). Since new IA cartridges which had undergone the same procedure did not show detectable clenbuterol by this MS method, it was assumed that trace amounts of clenbuterol from previous usage(s) might have been retained in the cartridge and contaminated the succeeding analyses. As the mass spectrometric method is at least ten times more sensitive than the conventional UV detection method, regenerated IA cartridges previously checked to be 'clean' by UV detection may not be suitable for the MS method. More vigorous cleaning and thorough checking procedures need to be implemented if IA cartridges are to be reused in this technique. Nevertheless, this serves as a good example to illustrate the capabilities of the MS method to detect trace levels of this veterinary drug in real samples. Figures 4 and 5 show SIM mass chromatograms from a sample of beef liver and a sample of minced beef, respectively, both spiked with 1

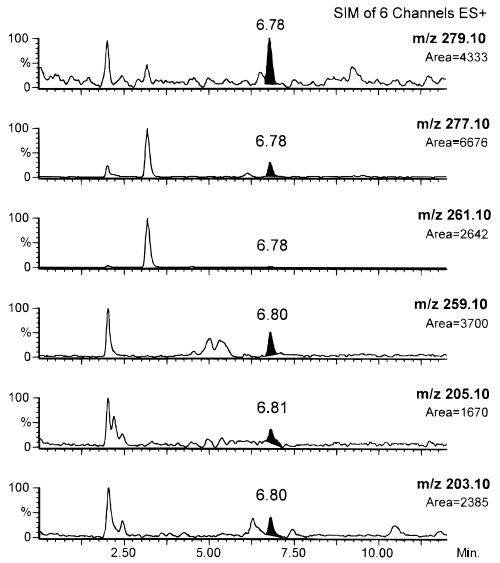


Figure 4. LC/ESMS/SIM mass chromatograms of a beef liver sample spiked with 1.0 ppb of clenbuterol and cleaned up with a new IA cartridge. Experimental conditions as in Fig. 2. Mass chromatograms were normalized to the largest peak in each mass channel.

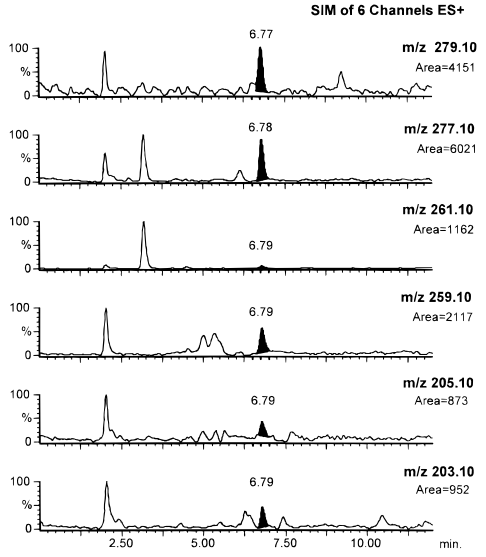


Figure 5. LC/ESMS/SIM mass chromatograms of a minced beef sample spiked with 1.0 ppb of clenbuterol and cleaned up with a new IA cartridge. Experimental conditions as in Fig. 2. Mass chromatograms were normalized to the largest peak in each mass channel.

ppb of clenbuterol and processed with new IA cartridges. One should be aware that although CID fragmentation process in the ion source region is commonly used to produce structurally characteristic fragments especially for single-analyzer low-cost instruments (so-called 'poor man's tandem mass spectrometry'), there is a risk that other compounds (with different molecular masses) that co-elute with the target compound and produce the same fragments as the analyte may interfere with the analysis. From Figures 4 and 5, it appears that IA chromatography is highly effective in removing interfering compounds, allowing very low levels of clenbuterol to be detected by this LC/MS/SIM method.

Multiple reaction monitoring (MRM)

It has been well established that MS/MS is one of the best approaches to improve specificity. Figure 6 shows the CID fragment ion mass spectrum of the protonated molecular ion at m/z 277 at a collision energy of 15 eV and collision gas (argon) pressure of 2.5×10^{-3} mbar.

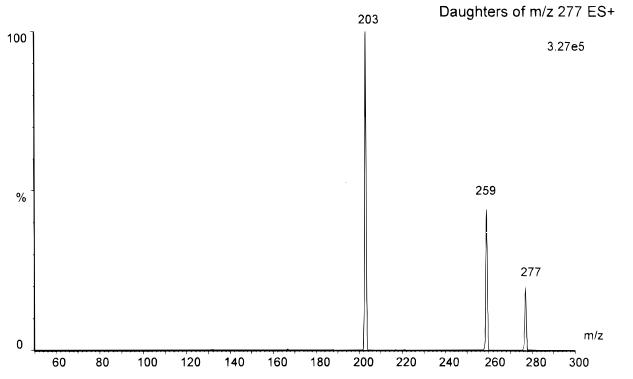


Figure 6. Fragment ion mass spectrum of clenbuterol at a collision energy of 15 eV and a collision gas pressure of 2.5×10^{-3} mbar. A 5 ng amount of clenbuterol was introduced into the electrospray ion source through flow injection.

Similarly to the CID fragmentation in the ion source region (Fig. 1), only two major fragments, $[M + H - H_2O]^+$ at m/z 259 and $[M + H - H_2O - C_4H_8]^+$ at m/z 203 (base peak), were observed. Doerge et al.[13] reported a different set of MS/MS fragment ions (m/z 221, 203, 161, 132 and 57) produced by APcI. They specifically pointed out that the m/z 259 and 168 fragment ions were not observed and suggested these two ions were not derived from the $[M + H]^+$ ions. Although we also did not detect the m/z 168 fragment ion, their former observation is contradictory to our findings. It is not sure why there is such a large discrepancy between our results and theirs, despite the fact that both laboratories were using the same type of MS/MS instrument (in Ref. 13 there is no indication of what instrument was used for the MS/MS experiments but a later publication[14] suggested that a Quattro II triple quadrupole mass spectrometer might have been used).

The relative intensity ratios between two fragment ion peaks as shown in Fig. 6 varied according to the collision energy and collision gas pressure. At a fixed collision gas pressure of 2×10^{-3} mbar, the optimum collision energies to yield the highest response for these two fragment ions (m/z 259 and 203) were 11 and 17 eV, respectively. The relative intensity ratio between fragment ion m/z 259 and 203 peaks is $\sim 1:3$. If the collision energy is fixed at 15 eV for both ions, the corresponding ratio will become 1:7. For MRM, a lower cone voltage of 25 V was used to maximize the abundance of the parent ion (m/z 277) in the ion source region. In order to maximize sensitivity, collision energies for each parent/fragment ion pairs (11 eV for m/z $277 \rightarrow 259$ and 17 eV for m/z $277 \rightarrow 203$) were programmed within each MRM mass switching cycle through the MassLynx acquisition software. MRM mass chromatograms from an injection of 50 pg clenbuterol standard acquired under these conditions are shown in Fig. 7. The background chemical noise was considerably lower than in the single MS case (SIM). If confirmation criteria require both ion pairs (m/z $277 \rightarrow 203$ and m/z $277 \rightarrow 259$) to be positive (signal-to-noise ratio $>3:1$), the MDLs would be around 10 pg per injection or equivalent to 0.07 ppb in meat (based on a 10 μl injection from 0.42 ml of final extract and a 6 g sample). Again, the MDL can be proportionally lower if a larger injection volume or more concentrated extract is used. Calibration graphs showed linear responses over three orders of magnitude (from 10 pg to 10 ng; r = 0.9999 m/z for m/z $277 \rightarrow 203$ and 0.9998 for m/z $277 \rightarrow 259$).

In our later experiments, we confirmed the observation of Debrauwer and Bories[11] that the addition of ammonium acetate buffer to the mobile phases has an adverse effect on the sensitivity. By removing the ammonium acetate from our mobile phases and also reducing the formic acid concentration to 0.05% (the pH remained at ~ 3), the sensitivity was improved by a factor of 3.

Figure 8 shows an injection of 10 pg of clenbuterol standard using 0.05% formic acid in both mobile phase A (water) and B (acetonitrile) and under the same gradient programming. There was a slight upward shift of the retention time but the chromatographic integrity was still maintained. This clearly demonstrates that as

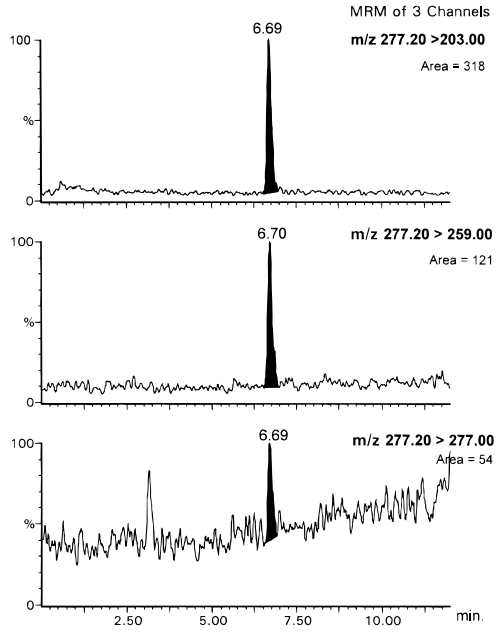


Figure 7. LC/ESMS/MS multiple reaction monitoring mass chromatograms from an injection of 50 pg of clenbuterol standard under LC conditions as described in the Experimental section. Ammonium acetate was present in the mobile phases. The parent ion (m/z 277) transmission and two CID fragment ions (m/z 259 and 203) from the $[M + H]^+$ ions were monitored.

low as 3–5 pg of clenbuterol (equivalent to 0.02–0.04 ng g^{-1} of sample) can be detected. Unfortunately, our main analyses were performed based on the ammonium acetate-containing mobile phases (as described in the Experimental section) and have not yet taken the advantage of this new improvement.

Figures 9 and 10 show MRM chromatograms of a 1 ppb spiked beef liver and a 1 ppb spiked minced beef sample, respectively, both of which were cleaned up with unused IA cartridges. High specificity is demonstrated as the two fragment ion traces (m/z 277 → 203 and m/z 277 → 259) show only the clenbuterol peak at a

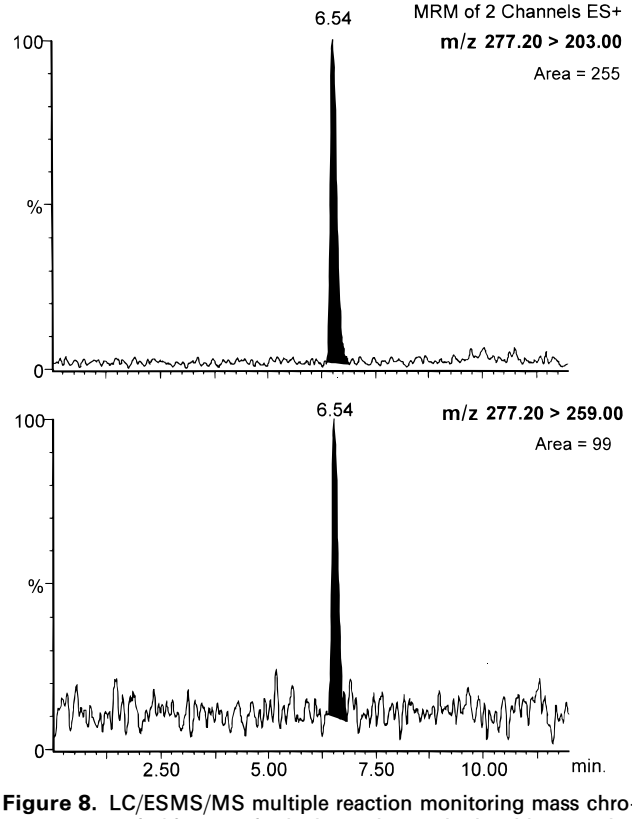


Figure 8. LC/ESMS/MS multiple reaction monitoring mass chromatograms of 10 pg of clenbuterol standard without using ammonium acetate in the mobile phases. Only two CID fragment ions (m/z 259 and 203) from the $[M + H]^+$ ions were monitored.

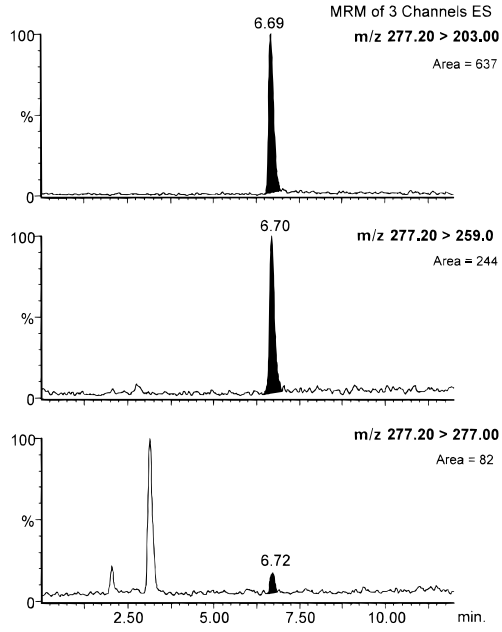


Figure 9. LC/ESMS/MS multiple reaction monitoring mass chromatograms of a blank beef liver sample spiked with 1 ppb of clenbuterol. Data were acquired under the same conditions as in Fig. 7. Mass chromatograms were normalized to the largest peak in each mass channel.

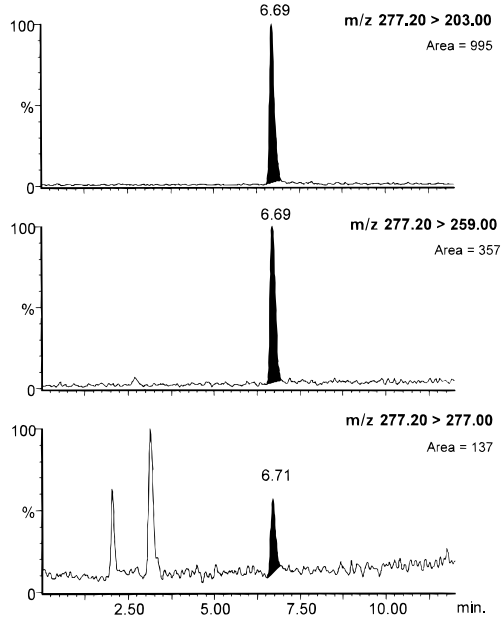


Figure 10. LC/ESMS/MS multiple reaction monitoring mass chromatograms of a blank minced beef sample spiked with 1 ppb of clenbuterol. Data were acquired under the same conditions as in Fig. 7. Mass chromatograms were normalized to the largest peak in each mass channel.

retention time of 6.7min and a consistent relative intensity ratio of ~2.7. The transmission of the parent ion (m/z 277) was also monitored and its relative intensity ratio (~14% of fragment ion m/z 203) served as an additional confirmation criterion.

Quantification in the current methods (LC/MS and LC/MS/MS) is based on the external standard method, which may be susceptible to fluctuations in the operating conditions and matrix effects. We have not thoroughly investigated whether different matrices will have any adverse effect on the accuracy of quantification. Results from our spiking study (at 1 and 5 ppb) indicated good agreement between the measured and expected concentrations. It is believed that by keeping the injection volume to a minimum (10 μl in our case) and using a 2 μm particle size LC packing material to facilitate improved chromatographic separation (with the additional advantages of fast speed and less solvent consumption), reasonably accurate quantification will result. However, when a stable isotopically labeled internal standard is available, it is always preferable to use the internal standard method.

CONCLUSION

This paper has demonstrated excellent sensitivity and specificity provided by the powerful combination of immunoaffinity chromatography and on-line LC/MS and LC/MS/MS with electrospray ionization in the determination of sub-ppb levels of clenbuterol in biological samples. Immunoaffinity chromatography is a simple, specific and highly efficient sample clean-up method producing extracts highly suitable for conventional LC/UV, GC or more sophisticated instrumental analysis such as GC/MS and LC/MS. LC/ESMS, on the other hand, provides excellent sensitivity and specificity for the determination of clenbuterol without chemical derivatization. The LC/ESMS/SIM method is readily applicable to most low-cost single mass analyzer LC/MS instruments. Proper control of the sampling cone voltage in the ion source region produces diagnostic ions for identification. Under certain criteria, such as LC retention time and relative intensity ratios among ions being monitored, the method is considered to be specific. However, if even higher specificity is required, MS/MS would be the best choice. Since most triple-quadrupole instruments have a high ion transmission efficiency, the absolute sensitivity loss resulting from switching to the MS/MS mode is easily offset by the great reduction in chemical background noise resulting in considerably improved overall signal-to-noise ratios, thus increasing the detectability of the analytes. The present study concentrated on the determination of clenbuterol, but it is expected to be equally valid for other β-agonists.

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