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Rapid analysis of β-agonists in urine by thermospray tandem mass spectrometry

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Abstract

A method is described for the analysis of β-agonists in urine of cattle. The method uses solid-phase extraction (SPE), followed by analysis of the resulting extract by flow injection thermospray tandem mass spectrometry (TSP-MS–MS). Sample preparation is performed using a mixed-bed SPE procedure using a sorbent having both hydrophobic and ionic properties. MS–MS analysis following thermospray ionization, is performed in single-reaction monitoring parent mode. In that way isotope dilution can be used for quantitation of clenbuterol. Data are presented on precision and accuracy for clenbuterol and related compounds. Furthermore, data acquisition was performed in full-scan neutral loss mode to indicate the suitability of flow injection analysis (FIA)–TSP-MS–MS for exploratory analysis. Detection of β-agonists in this mode is based on the presence of the N-*tert.*-butyl-β-ethanolamino moiety and, in that respect, detection of known as well as unknown compounds having this moiety will take place. This feature is exemplified by the analysis of samples containing several compounds.

1. Introduction

In the EC the use of β-agonists as growth promoting agents in the fattening of animals for human consumption is banned. Nevertheless these compounds are frequently found in the urine of cattle and analysis for the purpose of regulatory control is carried out in most, if not all, countries. Besides regulatory control, there is a growing interest in methods suitable for "real-time" analysis to perform process control. Within the FLAIR (Food-Linked Agro-Industrial Research) Concerted Action nr. 8 this subject was studied extensively and part of the presented work was carried out within the framework of this EC project.

Furthermore, an additional analytical problem is the use of slightly modified compounds for growth promotion. These compounds are usually not detected whenever a target-compound approach is applied.

For regulatory control, the analysis is usually carried out using GC–MS in the multiple ion detection (MID) mode [1,2]. Although a very effective technique, it has three major drawbacks: the compounds are targeted, so related compounds will not be detected, derivatization is usually necessary and this may impart unwanted selectivity and variability and, furthermore, the procedure typically takes two days to carry out.

Whenever the results of analysis should be

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available on a short term, as for "real-time" analysis, GC–MS methods generally are not very suitable. In that case application of LC–MS–MS techniques may offer better performance.

We present here a flow injection (FIA)–TSP-MS–MS method that may be used in either of two ways: (a) a method for semi-quantitative target-compound analysis that is suitable for very rapid analysis of residues of β-agonists and (b) a method for rapid qualitative group-specific analysis of nanogram amounts of known as well as unknown β-agonists.

2. Experimental

2.1. Materials and instrumentation

All reagents used were of analytical grade. The absence of β-agonists in the blank urine used for spiking experiments was demonstrated using a method based on GC–MS with a limit of detection of 0.2 ng/ml [1].

Clenbuterol-d_6, the analogue of clenbuterol where six protons at the *tert.*-butyl moiety are substituted with six deuterons, was used as an internal standard, allowing quantitation by isotope dilution.

Sample preparation was performed using XtrackT columns XRDAF515 (World Wide Monitoring, Bristol, PA, USA) containing 500 mg of a sorbent having ionic and hydrophobic properties. The clean-up procedure was adapted from the procedure published by Montrade et al. [3]. In brief, samples are hydrolyzed using glucuronidase/arylsulfatase from *Helix pomatia* at pH 4.8, using acetate buffer for pH adjustment. Following hydrolysis, the pH is adjusted to 6.0 using 0.1 *M* phosphate buffer and the extract is applied to a column conditioned with 3 ml each of methanol, water and 0.1 *M* phosphate buffer. The column is washed with 0.1 *M* phosphate buffer and the analytes are eluted with methanol containing 3% concentrated ammonium hydroxide.

The analysis is carried out using a Finnigan MAT TSQ70 (San Jose, CA, USA) mass spectrometer equipped with a thermospray II interface. The interface is operated at 200°C block temperature and 90°C vaporizer temperature. The repeller was at 80 V, no discharge ionization was used. Argon was used as collision gas at a pressure reading of 1.2 mTorr and a collision offset of −13 eV was applied. The carrier eluent consisted of 30% methanol in water with an overall concentration of 0.05 *M* ammonium acetate. The solvent was pumped at a flow-rate of 1.0 ml/min by a Gilson 305 pump (Villiers-le-Bel, France) equipped with an additional pulse damper. Aliquots of 50 μl were injected directly into the carrier eluent by means of a Gilson 231-401 autosampler.

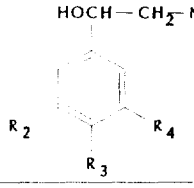
2.2. Data acquisition

To perform semi-quantitative analysis, a procedure for single reaction monitoring (SRM) in parent mode was used, monitoring the loss of water (M_r 18) and methylpropene (M_r 56) from the molecular ions. For cimaterol and clenbuterol-d_6 corresponding losses were monitored (Table 1). By applying parent mode data acquisition, the data system will record the masses of the precursor ions rather than the masses of the product ions. In this way the use of clenbuterol-d_6 as an internal standard becomes possible regardless of the fact that the mass of the product ion monitored is the same as for unlabelled clenbuterol.

Urine samples were spiked with five β-agonists (cimaterol, clenbuterol, terbutaline, mabuterol and salbutamol) at 1, 2 and 5 ng/ml, respectively. Aliquots of 5 ml of urine each from each spiking level were analyzed in triplicate. Following clean-up, to each extract 15 ng of clenbuterol-d_6, corresponding to 3 ng/ml in the urine, was added as an internal standard. The extracts were evaporated to dryness and redissolved in 0.50 ml of carrier eluent. Subsequently the extracts were analyzed using FIA–TSP-MS–MS in SRM parent mode. Aliquots of 50 μl, corresponding to 0.5 ml of urine, were injected in duplicate.

To perform group-specific qualitative analysis, data acquisition is performed in full-scan neutral

Table 1
Molecular structure and SRM data for the investigated analytes

Name					SRM
	R	R	R_3	R_4	
Clenbuterol (CLEN)	$C(CH_3)_3$	Cl	NH_2	Cl	277→203
Clenbuterol-d_6 (CLEN-d_6)	$C(CH_3)_2CH_2$	Cl	NH_2	Cl	283→203
Mabuterol (MAB)	$C(CH_3)_3$	Cl	NH_2	CF_3	311→237
Terbutaline (TER)	$C(CH_3)_3$	OH	H	OH	226→152
Salbutamol (SAL)	$C(CH_3)_3$	CH_2OH	OH	H	240→166
Cimaterol (CIM)	$CH(CH_3)_2$	CN	NH_2	H	220→160

loss mode. The scanning range of the first quadrupole is from 200 to 370 amu at one scan per second. Consequently the scan range of the second quadrupole is from 126 to 296 amu.

3. Results and discussion

3.1. Semi-quantitative determination

Fig. 1 shows a typical result for the analysis of urine samples spiked at 1 ng/ml. Table 2 presents the corresponding quantitative data of the three spiking levels tested. From Fig. 1 it is clear that detection of all analytes except salbutamol is readily performed at 1 ng/ml level with sufficient signal-to-noise ratio. For salbutamol, signal-to-noise ratio is low and, although this compound is detected, the limit of detection (LOD) is equal to 1 ng/ml. For the other compounds, based on signal-to-noise ratio, a LOD of at least 0.5 ng/ml in the urine is achievable. Reagent blanks do not indicate the presence of any of the analytes. Analysis of the blank urine extract, however, seems to indicate the presence of cimaterol and clenbuterol. For cimaterol this is most likely caused by interfering compounds, because all urine blanks analyzed during this study show a comparable blank offset. These interferences were not characterized any further. Consequently, for cimaterol the clean-up has to be improved or, alternatively, chromatographic separation of the analyte and the interference may be applied. For clenbuterol, other urine blanks did not show an offset, so for this compound it may have been incidental contamination. However, especially for clenbuterol the signal is rather small and quantitation yields a blank offset of approximately 0.3 ng/ml.

Quantitation is carried out by using clenbuterol-d_6 as an internal standard. For clenbuterol the quantitative results are therefore acceptable, especially when taking into account that clenbuterol-d_6 is only added after clean-up

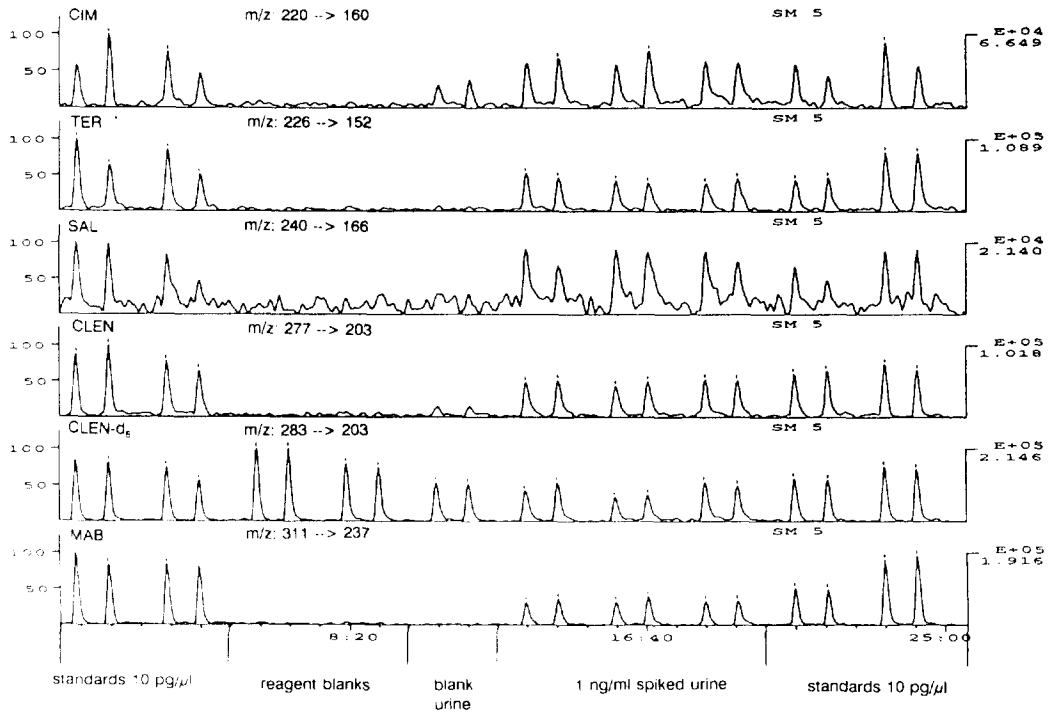


Fig. 1. SRM profiles for the FIA–TSP-MS–MS analysis of urine samples spiked at 1 ng/ml with five β-agonists.

so recovery losses are not corrected. For the other compounds, quantitative results should be improved. Repeatability is again acceptable for clenbuterol and perhaps for mabuterol and terbutaline, but also here improvements have to be made. However, as the use of these compounds in the fattening of animals for human consumption is banned within the EC, indication of the presence itself is more important than accurate quantitative results. Improvement of quantitation may be achieved by using isotope-labelled standards added before clean-up. Particularly for salbutamol the use of salbutamol-d_6 as an internal standard could improve quantitation. The results of analysis justify the statement that the presented method yields at least semi-quantitative information, except for salbutamol.

Together with the clean-up, not including hydrolysis, the entire procedure, including calibration, can be performed within two hours for a

Table 2
Results for the analysis of clenbuterol (CLEN), salbutamol (SAL), cimaterol (CIM), terbutaline (TER) and mabuterol (MAB) in spiked urine samples purified on XtrackT SPE columns

Spike level	CLEN		SAL		CIM		TER		MAB	
(ng/ml)	Mean (ng/ml)	C.V. (%)	Mean (ng/ml)	C.V. (%)	Mean (ng/ml)	C.V. (%)	Mean (ng/ml)	C.V. (%)	Mean (ng/ml)	C.V. (%)
1.0	1.1	15	1.6	28	1.5	25	1.0	21	0.7	21
2.0	2.4	18	4.3	31	2.7	19	1.9	9	1.5	18
5.0	4.4	12	12.2	19	5.9	7	5.6	14	2.9	8

set of 20 samples. In that respect the method is far more rapid than any GC–MS method now available.

3.2. Qualitative determination

Tandem mass spectrometry may also be used for exploratory analysis to detect the possible presence of unknown compounds. Using neutral loss scanning, the loss of 74 amu can be monitored, corresponding to the loss of water followed by the loss of methylpropene from the molecular ion. It is assumed that for all N-*tert.*-butyl substituted β-ethanolamines, this fragmentation will occur when using alike experimental parameters. For all known compounds this has been confirmed in our laboratory.

Fig. 2 shows the analysis of a 50-μl aliquot, corresponding to 0.5 ml of urine, of a urine extract spiked prior to clean-up, at 3 ng/ml, with several β-agonists. The presence of the peak at 4.4 min indicates the presence of one or more compounds that comply with the scanning requirement: the loss of 74 amu.

The inset in Fig. 2 shows the CID spectrum averaged over the peak at 4.4 min. This spectrum indicates the presence of salbutamol $[M+H]^+$ at *m/z* 240 and $[M+H-H_2O]^+$ at *m/z* 222, terbutaline $[M+H]^+$ at *m/z* 226, clenbuterol $[M+H]^+$ at *m/z* 277/279 and mabuterol $[M+H]^+$ at *m/z* 311/313. It is obvious that these compounds are readily detected at this concentration level. The presence of two fragment ions for salbutamol may be the reason for the low sensitivity in the semi-quantitative analysis, because there the loss of 74 amu from only one ion (*m/z* 240) is monitored.

To exemplify the detection of possible unknowns, a rather artificial experiment was carried out. Recently the abuse of the brominated analog of clenbuterol was discovered [4]. At that time, in our laboratory, GC–MS was used for the analysis. One of these samples was analyzed using the presented method. Experimental conditions were the same as in Fig. 2. Again an aliquot equivalent to 0.5 ml of urine was injected while scanning the mass spectrometer for the loss of 74 amu. Fig. 3 indicates the presence of compound(s) losing 74 amu, and the averaged

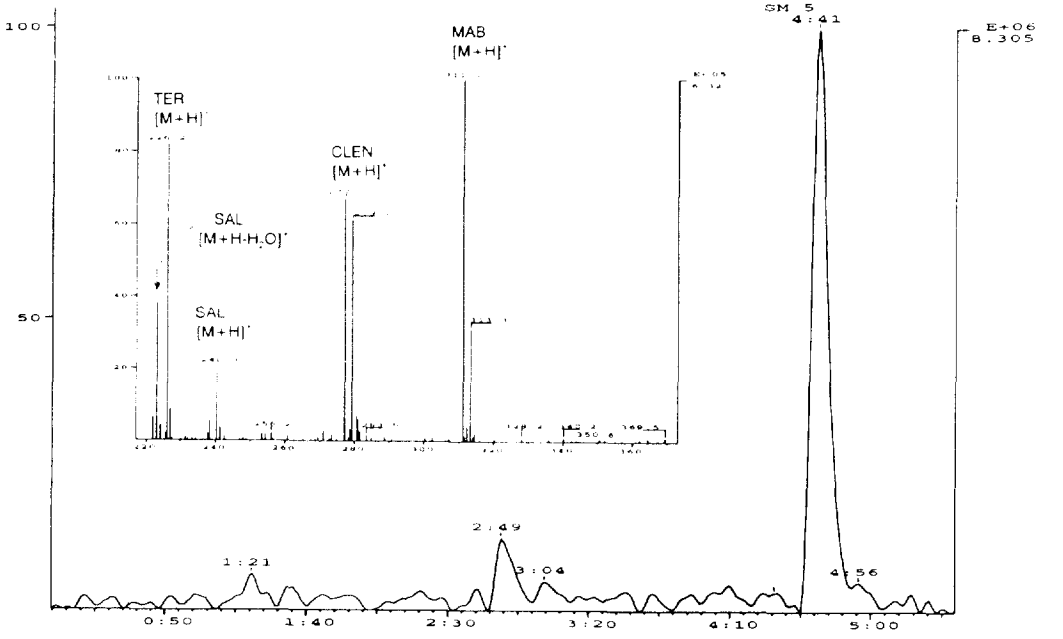


Fig. 2. Injection of an extract of a urine sample spiked at 3 ng/ml, indicating the simultaneous detection of all N-*tert.*-butyl substituted compounds present. The mass spectrometer was operated in full-scan neutral loss (74 u) mode.

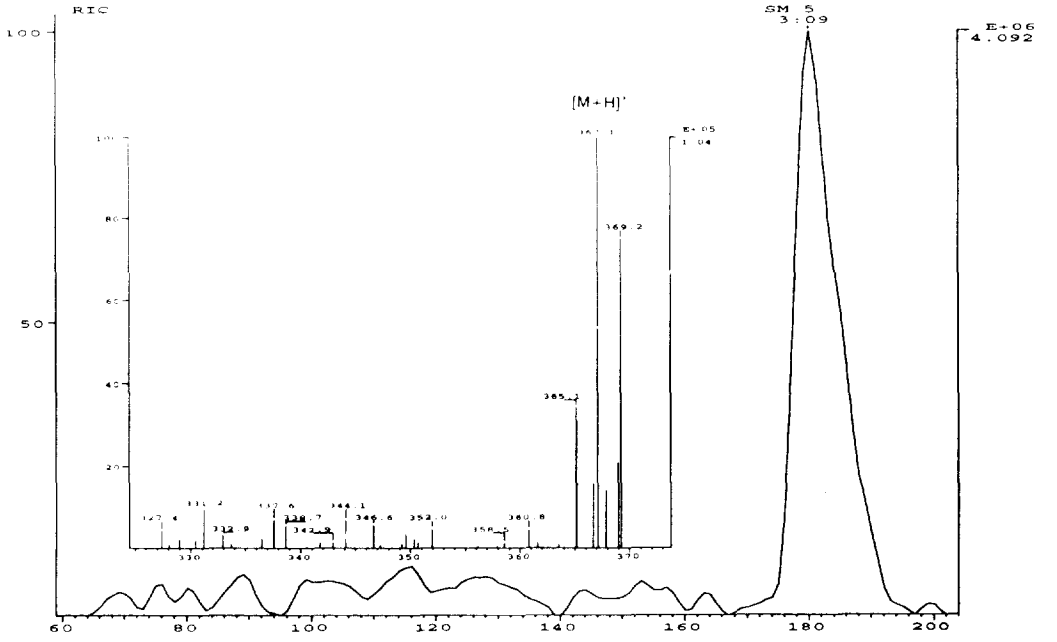


Fig. 3. Injection of an extract of a urine sample exemplifying the detection of a possible unknown compound. The CID spectrum reveals the presence of bromobuterol. The amount was estimated at 0.7 ng/ml in the urine.

CID spectrum reveals the presence of a compound containing two bromine atoms with a mono-isotopic molecular mass of 364, $[M+H]^+ = 365$. This compound was previously identified as bromobuterol, the brominated analog of clenbuterol. Using GC–MS, the amount was estimated at 0.7 ng/ml assuming a response per mass unit equal to clenbuterol.

From the above examples, the possibilities of tandem MS for group-specific detection, in this case specific for N-*tert.*-butyl substituted β-ethanolamines, are apparent even for low concentration levels like 1 ng/ml, although this strongly depends on the compound. It should be emphasized, however, that the loss of 74 amu may not be an exclusive feature of N-*tert.*-butyl substituted β-ethanolamines alone, so interference may occur. Only the analysis of a large number of samples from as diverse origin as possible will indicate whether or not the specificity is sufficient. Currently this item is under investigation.

4. Conclusion

The presented method may be used for rapid analysis of several β-agonists in urine in a semi-quantitative fashion at sub-ng/ml levels. Analysis is rapid but specificity should be studied more extensively. Operating the mass spectrometer in full-scan neutral loss mode, the possibilities of tandem mass spectrometry for group-specific detection of structure-related compounds are indicated. Detection of an unknown as well as known compounds at ng/ml levels is demonstrated.

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