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Confirmatory analysis of clenbuterol using two different derivatives simultaneously

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ABSTRACT

To meet the requirements of the European Community for confirmatory analysis of clenbuterol using low-resolution mass spectrometry, usually two different techniques (*i.e.* electron impact and chemical ionization) have to be applied to confirm unambiguously its presence in extracts of urine. This paper describes the application of two different derivatives and the simultaneous analysis of these two different derivatives in one gas chromatographic–mass spectrometric analysis. With the proposed combination of techniques, Community requirements can more easily be met in only one analytical run. Examples of the analysis of some urine samples are presented, as well as data on linearity, repeatability and equivalence of the combined technique to separate determinations.

INTRODUCTION

European Community (EC) requirements for confirmatory analysis of *e.g.* growth-promoting agents like clenbuterol (4-amino-3,5-dichloro-[(*tert.*-butylamino)methyl]benzyl alcohol) state that, when using low resolution mass spectrometry (LRMS), identification of the analyte has to be based on preferably at least four characteristic fragment ions. When these four ions are not present using a single technique, such as electron impact ionization (EI), a second technique, *e.g.* chemical ionization (CI), should be used. Both techniques should yield two diagnostic ions each, to obtain a total of four fragment ions for identification. Alternatively, different derivatives can be used [1].

Clenbuterol is a powerful growth promoting agent and, in the Netherlands, the use of this substance in the fattening of cattle for human consumption is forbidden. Nevertheless, it is frequently found in the urine of cattle. The mass spectrometric behaviour of clenbuterol on EI is troublesome in terms of the necessity to meet the EC demands for confirmation; only one major fragment ion is obtained, at m/z 86, corresponding to fission of the C–C bond in the β-ethanolamino part of the molecule. Other fragment ions are obtained at m/z 243, 262, 277 and 333 [2], of which m/z 262 is only *ca.* 10% of the base peak and the other ions are all of even lower intensity. As the sensitivity of the determination is mainly determined by the least intense fragment ion that is incorporated in the measurement, the lack of high-intensity fragment ions seriously decreases the lower limit of detection. Furthermore, because these low-intensity ions cannot always be measured accurately, a second analysis using another technique, such as CI, is frequently necessary to obtain four diagnostic ions suitable for unambiguous identification.

This paper describes the application of two different derivatives, *i.e.* trimethylsilyl (TMS) and

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tert.-butyldimethylsilyl (tBDMS), for the identification of clenbuterol at ng/ml levels. This combination can be used to obtain four high-intensity diagnostic ions in one gas chromatographic–mass spectrometer (GC–MS) analysis. An additional advantage is that the tBDMS derivative is reported to be much less prone to hydrolysis than the routinely used TMS derivative [3].

The method was applied to the analysis of some urine samples, which were also analysed using the procedure common in our institute with successive EI and CI measurements. Furthermore, data are presented concerning linearity and repeatability. The potential of the method for identification purposes is discussed with respect to EC requirements.

EXPERIMENTAL

Clenbuterol was purchased from Sigma (St. Louis, MO, USA). [$^2H_6$]Clenbuterol, used as an internal standard, was custom made. Stock solutions of the two substances containing 0.2 and 0.15 ng/μl were diluted immediately before use to obtain calibrant solutions containing 0.1, 0.2, 0.5, 1.0 and 2.0 ng/μl clenbuterol. [$^2H_6$]Clenbuterol was present in all calibrants at a concentration of 0.75 ng/μl. The stock solutions were stored at 4°C.

N,O-Bis(trimethylsilyl)trifluoracetamide (BSTFA) and N-methyl-N(*tert.*-butyldimethylsilyl)trifluoracetamide (MTBSTFA) were purchased from Pierce (Rockford, IL, USA). All reagents were of analytical grade.

GC–MS analyses in CI mode were carried out using a Finnigan MAT TSQ70 mass spectrometer (San Jose, CA, USA) equipped with a Hewlett Packard 5890 gas chromatograph (Avondale, PA, USA) and operated in multiple ion detection (MID) mode. Ammonia was used as reagent gas, and the pressure was adjusted to a reading of 8500 mTorr (1 mTorr = 0.133 Pa). GC separation was achieved on a DB5 capillary column (J&W Scientific, Folsom, CA, USA) (30 m × 0.25 mm I.D., film thickness 0.25 μm). The injector and interface were maintained at a temperature of 260°C. The GC oven was programmed from 110°C to 180°C at 4°C/min and then to 300°C at 30°C/min. EI experiments were carried out on a Finnigan MAT ITS40 ion-trap mass spectrometer equipped with a Varian gas chromatograph (Walnut Creek, CA, USA). The same type of capillary column and the same temperature settings were used.

Sample preparation

Clenbuterol was extracted from the urine using immunoaffinity chromatography and solid-phase extraction according to Schilt *et al.* [4]. Buffered urine was applied to an immunoaffinity column containing immobilized antibodies specific for clenbuterol. The column was washed, and then clenbuterol was eluted with methanol–acetic acid 0.1 *M*, pH 2.75 (70:30). The resulting extract was applied to a C_{18} cartridge column. The column was washed, and clenbuterol was eluted with methanol–acetonitrile (85:15).

The methanol extracts resulting from this clean-up procedure were divided into two equal aliquots of 1 ml each. The methanol was evaporated under nitrogen. One of the aliquots was derivatized using BSTFA to obtain the TMS derivative, the other was derivatized with MTBSTFA to obtain the tBDMS derivative. Derivatization with BSTFA was carried out by adding 100 μl of a 1:1 mixture of BSTFA and ethyl acetate to the evaporated extracts. This mixture was heated for 40 min at 60°C. Derivatization with MTBSTFA was performed by adding 100 μl of the pure derivatization reagent to the evaporated extracts followed by heating at 60°C for 60 min. After completion of the derivatization reaction, the reagent was evaporated under nitrogen and the residue was dissolved in ethylacetate. Corresponding sample aliquots were recombined, and the ethyl acetate was evaporated under nitrogen. The residue was then dissolved in 20 μl of toluene containing 5 ng/μl of PCB 138 (Promochem, Wesel, Germany) as a syringe standard. This procedure resulted in an equivalence of 0.125 ml of urine per μl extract for both derivatives. To avoid possible hydrolysis during storage, the extracts were analysed immediately following preparation.

RESULTS AND DISCUSSION

Electron impact

Fig. 1 shows a chromatogram and EI spectra of a standard solution containing both derivatives at a concentration corresponding to 1 ng/μl of clenbuterol. Both derivatives show a base peak at *m/z* 86, corresponding to fission of the C–C bond in the β-ethanolamine part of the molecule, with the charge remaining on the fragment containing the amine moiety. The corresponding fragments of $[M - 86]^+$ at *m/z* 262 and 264 for the TMS derivative and at *m/z* 304 and 306 for the tBDMS derivative are also present, but of fairly low intensity.

For confirmation purposes the $[M - 86]^+$ ions are not very useful because these ions are also present in the spectra of the internal standard [$^2H_6$]clenbuterol where they correspond to the equivalent fragmentation giving rise to $[M - 92]^+$ ions. The native clenbuterol and [$^2H_6$]clenbuterol derivatives are only partly separated (Δt_R = 1–2 s), and therefore relative intensities of these ions are offset by the presence of the internal standard. This offset is concentration dependent and, in many cases, will lead to erroneous results.

Chemical ionisation

Fig. 2 shows a chromatogram and CI spectra of a standard solution containing both derivatives at a concentration corresponding to 5 ng/μl of clenbuterol. Both derivatives show abundant quasi-molecular ions and only little fragmentation due to the loss of OH-TMS $[M + H - 90]^+$ or OH-tBDMS $[M + H - 132]^+$, respectively. Ion intensity ratios of *m/z* 349:351 and 391:393 were calculated as 100:69.9 and 100:70.8, respectively. Experimentally determined ratios are in good agreement with these values (Table I).

In contrast to the ions in the EI spectra, the quasi-molecular ions are well suited to confirma-

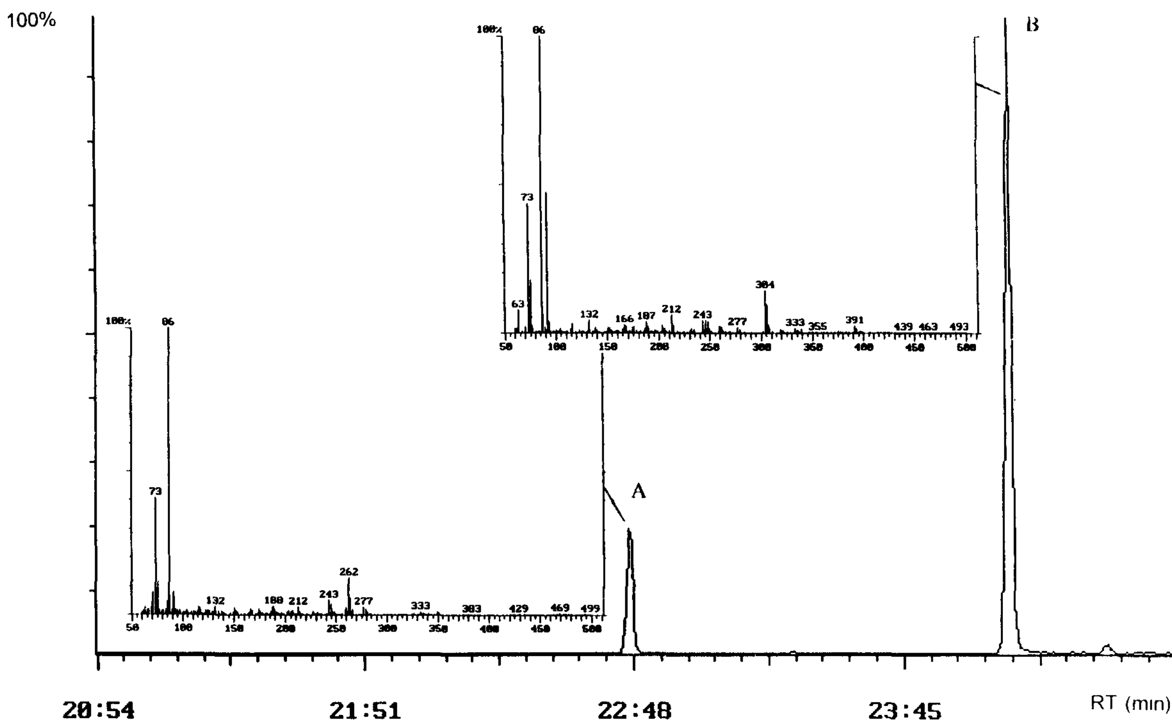

Fig. 1. Ion chromatogram and EI spectra of a standard solution containing 2 ng/μl each of (A) clenbuterol-1-TMS and (B) clenbuterol-1-tBDMS.

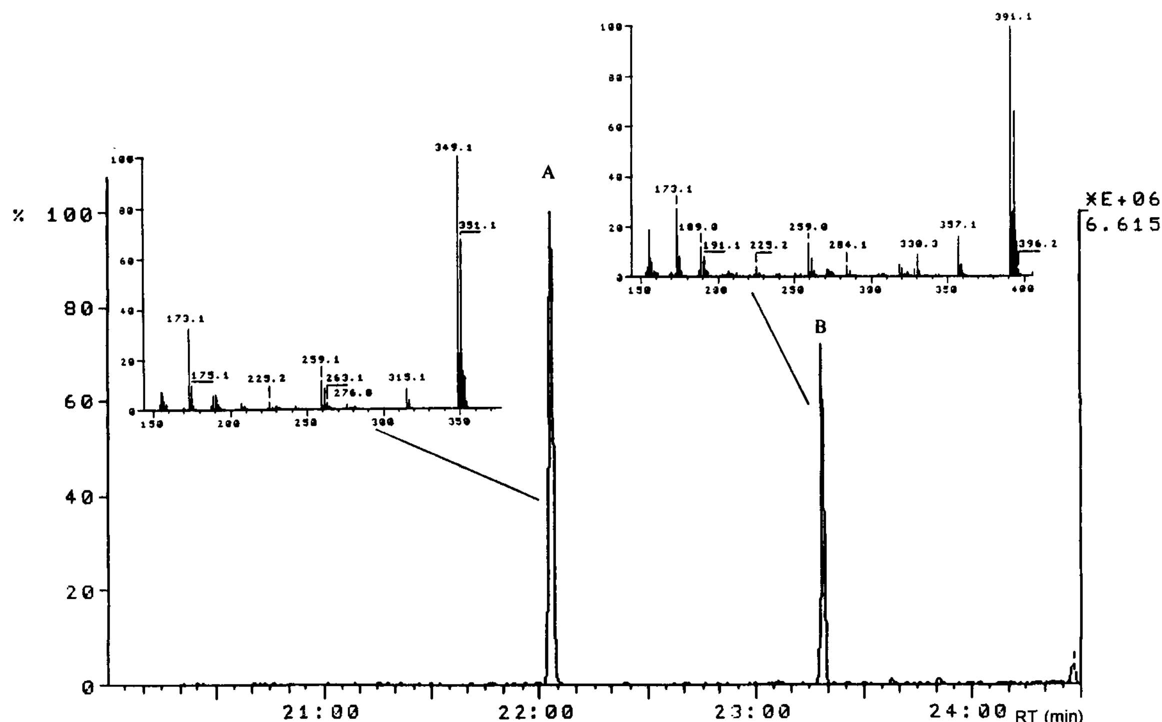

Fig. 2. Ion chromatogram and CI spectra of a standard solution containing 5 ng/μl each of (A) clenbuterol-1-TMS and (B) clenbuterol-1-tBDMS.

tory analysis: the ions are structure-related and abundant, and their ratio is well defined. Furthermore, no interference of the incompletely separated internal standard occurs. However, very high levels of the native compound can interfere the deuterium-labelled internal standard, owing to the presence of the $[M + 6]^+$ isotope peak with a relative abundance of *ca.* 0.7%. In general, this interference will not be significant, as long as the amount of native clenbuterol does

TABLE I

REPEATABILITY DATA ON THE SIMULTANEOUS ANALYSIS OF TWO CLENBUTEROL DERIVATIVES

Concentration, 0.5 ng/μl for each derivative; n = 10.

	TMS			tBDMS			*RRT*
	RRF	*m/z*		*RRF*	*m/z*		
		349	351		391	393	
Mean	0.906	100	67.8	0.834	100	68.5	1.057
S.D.	0.017	–	4.8	0.017	–	4.6	0
R.S.D (%)	1.9	–	7.1	2.1	–	6.7	0

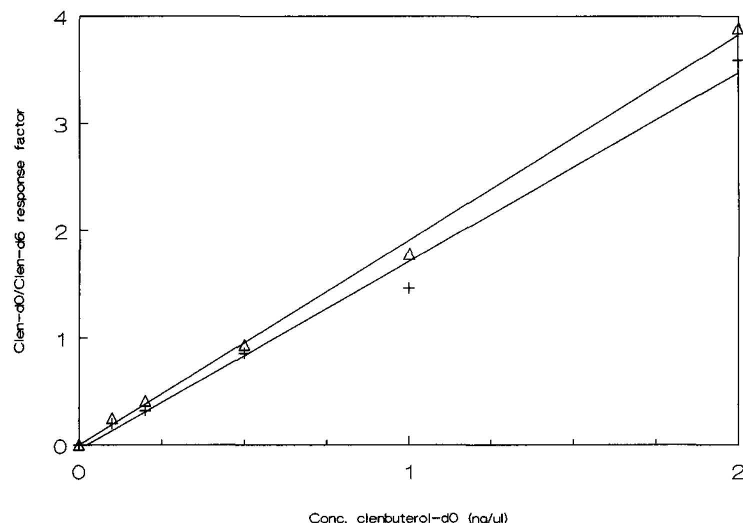

Fig. 3. Linearity plot of relative response *vs.* concentration for clenbuterol-1-TMS (△) and clenbuterol-1-tBDMS (+).

not exceed the amount of internal standard more than ten-fold.

The linearity was tested using calibrant solutions containing 0, 0.1, 0.2, 0.5, 1.0 and 2.0 ng/μl each of both derivatives. All solutions contained 0.75 ng/μl of [$^2H_6$]clenbuterol and 5 ng/μl of PCB-138 as a syringe standard. Dilution of the calibrant solutions was performed prior to derivatization. A calibration curve was constructed by means of relative response factors. Linearity proved to be good for both derivatives (Fig. 3). A typical chromatogram of a standard solution containing 0.2 ng/μl of clenbuterol for each derivative is shown in Fig. 4.

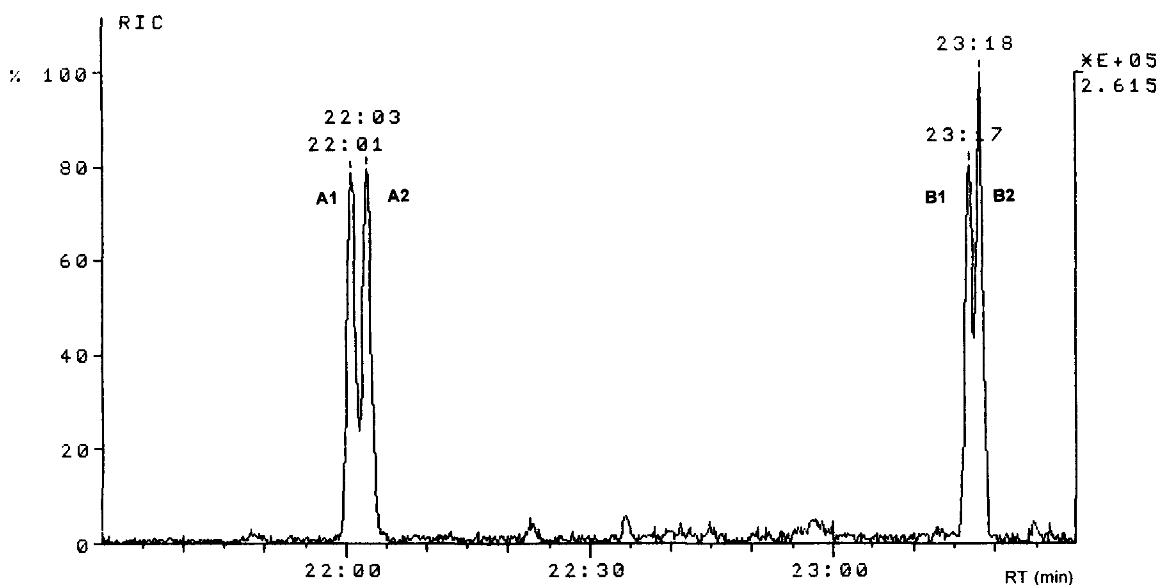

Fig. 4. Ion chromatogram of a diluted standard solution containing 0.2 ng/μl of clenbuterol and 0.75 ng/μl of [$^2H_6$]clenbuterol for both derivatives. Peaks: A1 = [$^2H_6$]clenbuterol-1-TMS: A2 = clenbuterol-1-TMS; B1 = [$^2H_6$]clenbuterol-1-tBDMS; B2 = clenbuterol-1-tBDMS.

TABLE II

COMPARISON OF RESULTS OF ANALYSIS OF URINE SAMPLES OBTAINED IN THE ROUTINE PROCEDURE AND FROM SIMULTANEOUS ANALYSIS OF TWO DERIVATIVES

Values are in ng/ml.

Urine samples	Routine procedure	Simultaneous analysis	
		TMS	tBDMS
Blank	–	–	–
Fortified (3 ng/ml)	2.7	3.0	3.2
Fortified (3 ng/ml)	2.4	2.8	2.8
1	0.66	0.74	0.62
2	8.8	7.6	8.8
3	13.5	11.7	11.6
4	17.6	16.0	15.4
5	2.6	2.6	2.6
6	41.9	44.5	39.3

The repeatability was tested by ten-fold injection of the 0.5 ng/μl calibrant solution. Results are presented in Table I. The relative retention time (*RRT*) is calculated as the retention time of clenbuterol-1-tBDMS relative to clenbuterol-1-TMS. The repeatability of both the response of the compounds and the ion ratios is good. Based on the acquired data, and assuming gaussian distribution, a maximum variation of 15% ($x \pm 2\sigma$) should be expected on the determination of relative ion intensities . This value is well within the limit of 20% mentioned in the EC directive [1] and is therefore acceptable for identification purposes.

Finally, the method was applied to some urine samples that had already been analysed using the routine procedure. The sample extracts, equivalent to 5 ml of urine, were split into two equal parts, and corresponding aliquots were derivatized with BSTFA and MTBSTFA, respectively.

Comparability of the results (Table II) is good (maximum deviation of 16%) and therefore simultaneous application of two different derivatives is advantageous. The limit for confirmatory analysis is estimated at 0.025 ng/μl at a signal-to-noise ratio of 10, corresponding to 0.2 ng/ml in the urine for both derivatives. The signal-to-noise ratio is somewhat better for the tBDMS derivative and, for this compound, a slightly better limit of detection can be achieved. Nevertheless, the determination of both derivatives is essential to confirm the presence of clenbuterol.

CONCLUSION

The method described is suitable for the confirmation of the presence of clenbuterol in urine samples in one GC run according to EC demands, *i.e.* measuring at least four diagnostic fragment ions. When CI is used, the selected quasi-molecular ions are all of high intensity and, as a consequence, reliable and sensitive detection is possible. Additional confirmatory information is obtained by the relative retention time of the two different clenbuterol derivatives. Results are comparable with the results obtained when two separate analyses using EI and CI are applied. The minimum concentration of clenbuterol neccessary to achieve confirmation according to the EC demands is in the order of 0.025 ng/μl, corresponding to 0.2 ng/ml in urine.

At present, investigations are aimed at extending this approach to a larger number of β-agonistic drugs. Attention is primarily focused on compounds that could be illegally used as growth promoting agent.

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