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Applicability of coupled-column liquid chromatography to the analysis of β-agonists in urine by direct sample injection
I. Development of a single-residue reversed-phase liquid chromatography–UV method for clenbuterol and selection of chromatographic conditions suitable for multi-residue analysis

A. Polettini[a], M. Montagna[a], E.A. Hogendoorn[b], E. Dijkman[b], P. van Zoonen[b,*], L.A. van Ginkel[b]

[a]*Institute of Legal Medicine, Laboratory of Forensic Toxicology, University of Pavia, Via Forlanini 12, I-27100 Pavia, Italy*
[b]*National Institute of Public Health and Environmental Protection (RIVM), P.O. Box 1, 3720 BA Bilthoven, Netherlands*

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Abstract

Optimisation procedures originally applied to coupled-column RPLC–UV for the residue analysis of polar pesticides were evaluated for the analysis of β-agonists in human and bovine urine using direct sample injection. Two approaches have been studied: (i) a multi-residue method (MRM) for the clean-up and separation of eight different β-agonists (isoprenaline, cimaterol, terbutaline, salbutamol, fenoterol, ractopamine, clenbuterol and mabuterol) and (ii) a single-residue method (SRM) focussed at the detection of clenbuterol residues in samples of urine.

Both approaches provided efficient procedures to process urine samples automatically with coupled-column LC. Particular attention was paid to selecting analytical conditions suitable for thermospray MS detection, which is to be investigated in the near future.

Though UV detection cannot offer enough selectivity for the simultaneous screening of a group of β-agonists, coupled-column RPLC–UV proved to be very powerful in SRM, allowing the detection of clenbuterol at the μg/l level in filtered (0.45 μm) human and bovine urine after direct sample injection.

1. Introduction

The illicit use of β-agonists in zootechnics and in sports is well documented [1–5]. In both cases the side effects of these drugs on protein synthesis and lipolysis are sought, although their stimulatory activity on respiration could also be exploited for doping purposes [6].

Many single-residue methods (SRMs) or multi-residue methods (MRMs) have been proposed for the analysis of β-agonists in biosamples based on immunoassay techniques [7–10], or involving high-performance liquid chromatographic (HPLC) [6,11–17] or gas chromatographic (GC)

* Corresponding author.

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separation [5,18–31] after purification of the analytes by means of either liquid–liquid partition [6,11,12,15–18,20,24,28], solid-phase extraction [5,13,17,21,23,30,31] or immunoaffinity chromatography [27,29,31,32]. The need of a confirmation defensible in the court of justice, i.e. based on mass spectrometric (MS) identification, and the availability of low-priced bench top GC–MS instruments explains the development of a large number of methods based on GC separation. Nevertheless, the use of GC is significantly limited by the high polarity of this class of compounds, requiring a derivatisation step prior to injection. Furthermore, the derivatisation procedures proposed for β-agonists are not entirely satisfactory owing to the low specificity of the resulting electron impact (EI) mass spectra (e.g. silyl derivatives) or to the restricted range of applicability (e.g. derivatives with boronic acids) [5,24]. This problem can be alleviated by using a very selective sample pretreatment technique such as immunoaffinity chromatography.

HPLC has significant advantages over GC for the analysis of β-agonists, the main ones being the lack of need for derivatisation and a good solvent compatibility of sample with the chromatographic system. However, its application was found to be limited because of the insufficient selectivity and/or sensitivity of common LC detectors on one hand, and difficulties of its interfacing with MS on the other. The recent development of effective HPLC–MS interfaces, such as thermospray or electrospray, however, opens new possibilities in the bioanalysis of these drugs [12,15,33,34]. Furthermore, as has been demonstrated in numerous publications, HPLC column switching offers the possibility to automate sample processing and to enhance sensitivity and selectivity by means of large volume injections and clean-up on the first column.

Recently, at the RIVM, optimisation procedures and strategies in method development have been derived for the determination of polar pesticides in various matrices using coupled-column RPLC and UV detection [35–40]. Basically, two method development approaches are used: MRMs for the simultaneous determination of a group of polar pesticides of widely different polarity in various types of samples [35–37], and SRMs for the rapid, sensitive assay of single analytes in aqueous environmental samples [38,39]. Both approaches make use of formulated criteria for important parameters such as clean-up, resolution, time of chromatographic run and (gradient) elution profile [36–40].

In this study the applicability of the SRM and MRM approaches for pesticides was investigated for the automated processing of β-agonists in urine samples including particular care in selecting analytical conditions suitable for thermospray (TSP) HPLC–MS, the desired technique in the bioanalysis of β-agonists which will be a matter of future investigation.

2. Experimental

2.1. Reagents

The β-agonists isoprenaline · HCl, cimaterol free base, terbutaline · H_2SO_4, salbutamol · H_2SO_4, fenoterol · HBr, ractopamine · HCl, clenbuterol · HCl and mabuterol free base, all with purity >99%, were obtained from the Laboratory for Residue Analysis (RIVM). HPLC-grade methanol and formic acid were purchased from J.T. Baker (Deventer, Netherlands). Ammonium acetate and triethylamine were from Aldrich (Axel, Netherlands) and Merck (Darmstadt, Germany), respectively. HPLC-grade water was prepared by purifying demineralised water in a Milli-Q system (Millipore, Bedford, MA, USA). For linear binary gradient elution water was used as solvent A and a mixture of methanol–water (95:5, v/v) as solvent B. Both solvents contained 0.1 *M* ammonium acetate, 0.17 *M* formic acid and 0.01 *M* triethylamine. Isocratic and step-gradient elutions were performed using mobile phases made of different proportions of methanol and water, containing 0.1 *M* ammonium acetate and 0.01 *M* triethylamine with (pH about 3.8) or without (pH about 7.5) 0.17 *M* formic acid.

Separate stock standard solutions (1 mg/ml expressed as free bases) were prepared in HPLC-

grade water containing 2.5% of methanol with the exception of isoprenaline and mabuterol which were dissolved in ethanol. For HPLC analyses dilutions were made in HPLC-grade water, with evaporation of ethanol under nitrogen flow when isoprenaline and/or mabuterol were added.

2.2. Equipment

LC instrumentation used for the experimental work consisted of the following components (see Fig. 1): an ASPI 232-401 autosampler, AS (Gilson, Villiers-le-Bel, France) equipped with a programmable high-pressure valve, HV (type 7010; Rheodyne, Cotati, CA, USA); a binary gradient pump, P-1, Model 250 from Perkin-Elmer (Norwalk, CT, USA) with a helium degassing system from Perkin-Elmer equipped with air-tight bottle connections from Omnifit (Cambridge, UK) to deliver mobile phases to the pumps under light pressure (60 p.s.i.; 1 p.s.i. = 6894.76 Pa); two isocratic Model 306 Gilson pumps, P-2 and P-3; one isocratic Model 305 Gilson pump, P-4; a two-valve switching device, MUST (Spark Holland, Emmen, Netherlands); a manometric module, MM (Model 805, Gilson). All flow-rates were set at 1 ml/min.

Detection (UV at 245 or 285 nm), and collection of UV spectra (190–370 nm) were performed on a photodiode array detector, D, Model 1000S from ABI (Foster City, CA, USA) equipped with a Kipp and Zonen (Delft, Netherlands) recorder.

Two 50 × 4.6 mm I.D. columns, packed with Microspher C_{18}, 3-μm particles, from Chrom-

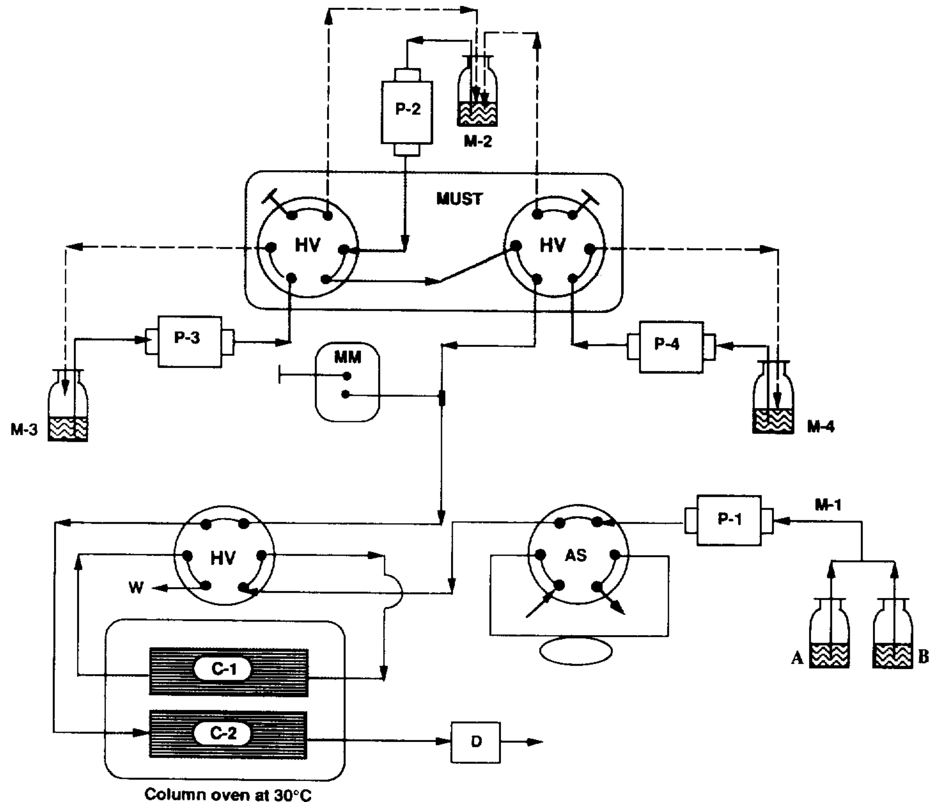

Fig. 1. Overview of the LC system used for experimental work. AS = Autosampler; HV = high-pressure valve; P-1 = binary LC pump; P-2, P-3, P-4 = isocratic LC pumps; MUST = two-valve switching device; MM = manometric module; C-1, C-2 = first and second separation columns; M-1, M-2, M-3, M-4 = mobile phases; D = UV photodiode array detector; W = waste.

pack (Middelburg, Netherlands) were used as first (C-1) and second column (C-2) for column switching experiments. A 10 × 3 mm I.D. guard column packed with 40-μm pellicular C_{18} material (Chrompack), was inserted before C-1. A 3-μm Microspher C_{18} column (100 × 4.6 mm) was used to establish gradient elution profiles without column switching. The columns were maintained at 30°C with a laboratory-made column oven connected to a circulating water system (Model 1441; Braun, Germany).

Calculations were performed with the program OPTIME Version 3.1 (RIVM) using a Macintosh MC II (Apple, Cupertino, CA, USA) personal computer.

2.3. Urine sample pretreatment

Human and bovine urine samples were filtrated on 0.45-μm Millex-HA (Millipore) filters prior to injection.

3. Results and discussion

3.1. General aspects

The β-agonists selected in the study for the MRM approach are listed in Table 1. The first step in the set-up of a coupled-column RPLC method is to select appropriate mobile and stationary phases [36–39]. In this case, special attention has to be paid to the physico-chemical properties of the analytes (basic or amphoteric, see Table 1) and also to the detection mode that will be applied to the presently developed procedure in a planned future study (TSP-MS). In fact, though there is good compatibility between RPLC and TSP-MS, a significant limitation is the necessity of a buffer in the mobile phase to ensure direct TSP ionisation ("buffer" ionisation). Furthermore, only volatile buffers such as ammonium acetate can be used to avoid interface clogging [41,42].

Taking into account this limitation, and based on preliminary results obtained earlier for the RPLC–TSP-MS analysis of β-agonists at the Institute of Legal Medicine of Pavia (data not published), mixtures of methanol and water containing ammonium acetate, formic acid and triethylamine (see Experimental) to reduce peak tailing of the analytes, were selected as mobile phase constituents.

Cartridge columns (50 × 4.6 mm I.D.) packed with 3-μm Microspher C_{18} were selected to achieve efficient separation, high sample load and on-line clean-up with column switching.

After the selection of the LC constituents, the UV spectra of the analytes were recorded and their retention behaviour was determined by constructing the second-order polynomial ln k vs. φ relations, where φ is the percentage of methanol in the mobile phase. The UV characteristics are listed in Table 1 and the retention behaviour is displayed in Fig. 2. As can be seen from this figure all individual analytes possess a proper RPLC retention ($1 < k < 10$) in various isocratic mobile phase composition. However, a satisfactory isocratic separation of all analytes is impossible and thus gradient elution is necessary. When linear gradient elution was applied, to both solvents A and B the same quantity of buffer constituents was added in order to avoid ions dilution in the mobile phase under gradient elution owing to the increment of the percentage of organic solvent, and thus to prevent possible disturbances on ionisation processes [42].

At this point our approach in method development is divided into SRM and MRM methodology, which will be discussed separately below.

3.2. SRM approach

One of the most favourable aspect of automated sample processing using LC–LC is the utilisation of the separation power of the first column (C-1). Beside the opportunity, in the case of aqueous samples, to enlarge sample injection volume for the improvement of the sensitivity, it offers the possibility to perform an efficient clean-up.

Another crucial feature of LC–LC is the transfer volume i.e. the time that C-1 is coupled on-line to the second column (C-2). In complex samples it is unavoidable that part of the interferences will be transferred together with the

Table 1
Names, structural formulae and UV characteristics of the β-agonists considered

Compound	Structural formula	λ_{max} (nm)	ϵ_{max} (l/mol · cm)
Isoprenaline		231	3200
		280	2600
Cimaterol		249	8500
		325	3700
Terbutaline		231	2900
		280	3000
Salbutamol		231	3000
		280	1500
Fenoterol		231	3100
		280	2900
Ractopamine		231	2700
		280	2000
Clenbuterol		245	7000
		300	2000
Mabuterol		245	9700
		300	2800

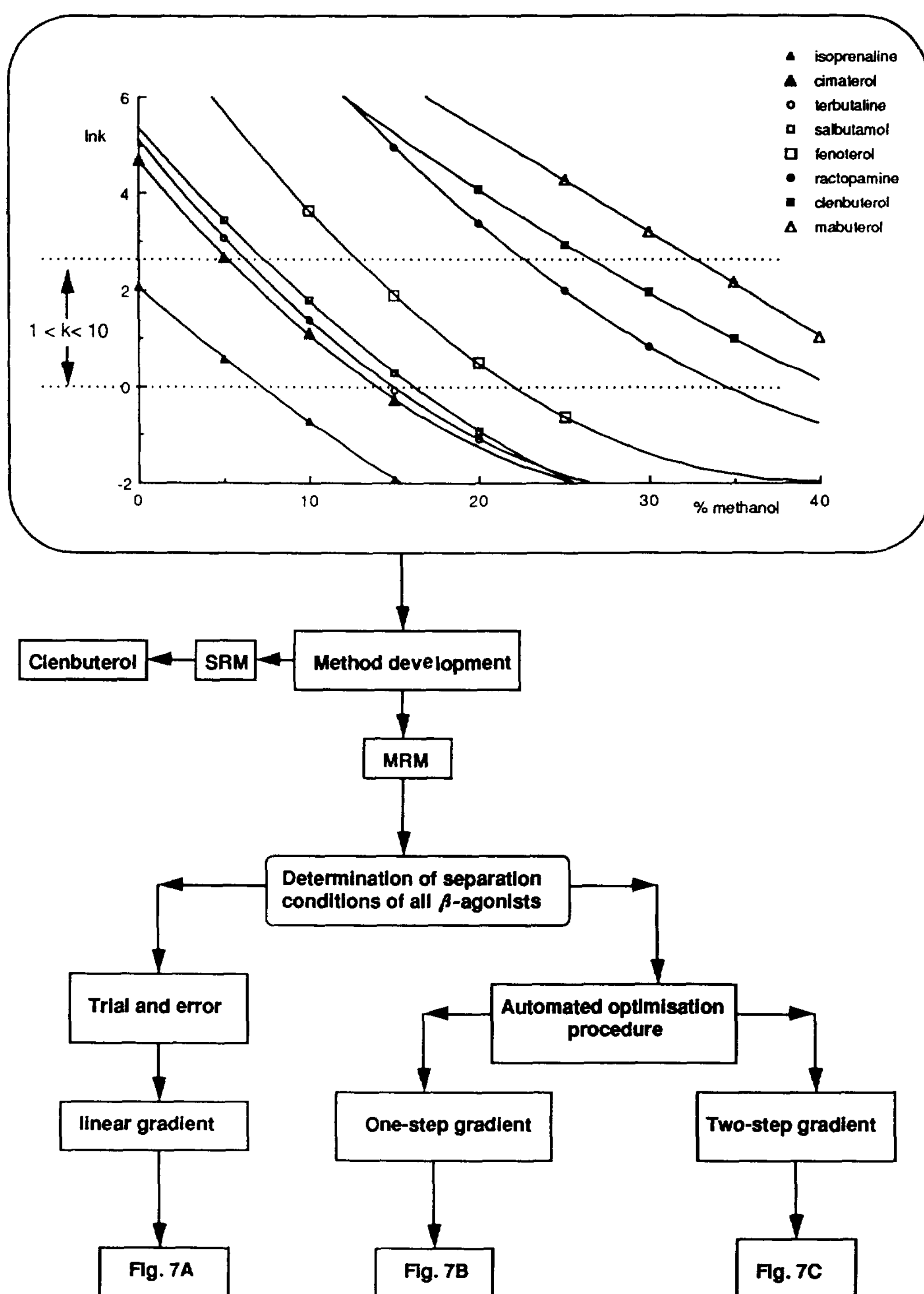

Fig. 2. Retention behaviour of the selected β-agonists on a 3-μm Microspher C_{18} column (50 × 4.6 mm I.D.) using methanol–buffer (0.1 *M* ammonium acetate, 0.17 *M* formic acid and 0.01 *M* triethylamine) as the mobile phase and flow path for the selection of adequate separation conditions (see Figs. 3 and 4).

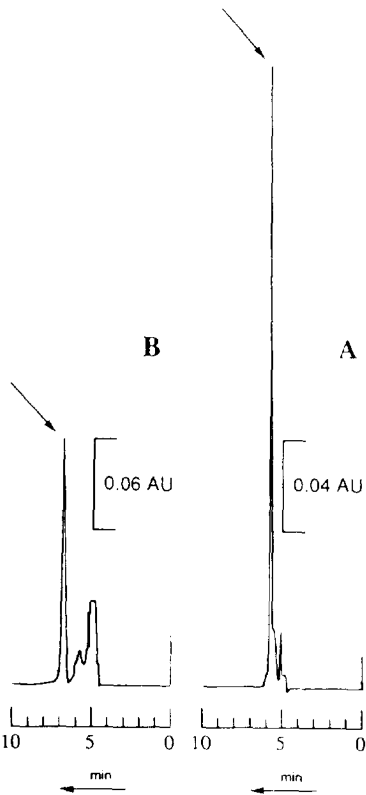

Fig. 3. Coupled-column RPLC–UV (245 nm) produced by the injection (150 μl) of blank human urine spiked with clenbuterol (10 μg/ml) with (A) and without (B) the addition of formic acid in the mobile phase. M-2 (40% of methanol in buffer, pH about 7.5) of C-2; M-1 = 25% methanol in buffer (pH about 3.8); clean-up volume, 3.90 ml; transfer volume, 0.50 ml. Clenbuterol is indicated by an arrow.

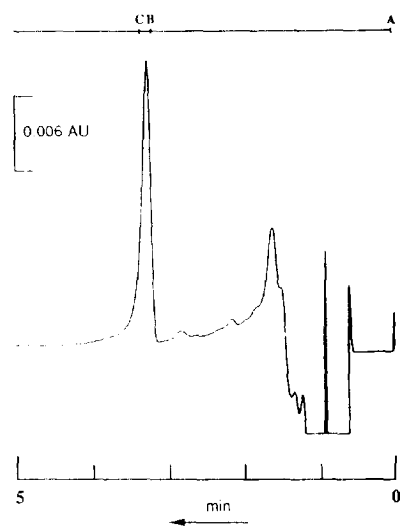

Fig. 4. RPLC-UV (245 nm) showing the elution of clenbuterol (150 ng in 1.5 ml of water) on C-1 for the establishment of column-switching conditions. Mobile phase M-1, 28.5% methanol in buffer (pH about 3.8). A = Injection of 1.5-ml sample; A–B = clean-up time; B = C-1 on-line C-2; B–C = transfer time; C = C-1 off-line C-2.

analytes. Hence, the attainable selectivity will be determined by (i) the effectiveness of clean-up on C-1 (clean-up volume) and (ii) the volume of the analyte fraction (transfer volume). In other words, optimal selectivity will be obtained in SRMs with the application of a minimal transfer volume. In pesticide residue analysis it has been clearly demonstrated [38,39] that coupled-column RPLC–UV provide sensitive, selective and moreover rapid and thus highly cost-effective SRMs. Hence it is very attractive to investigate the potential of this technique to this field of analysis.

Sensitivity and selectivity depend largely on the RPLC–UV properties of the analyte. In UV detection both selectivity (wavelength, nm) and sensitivity (ϵ, l/mol·cm) play a role; the analyte's C_{18} retention is important, since it influences both the maximum tolerable sample injection volume (sensitivity) and the potential for separation between analytes and early eluting interferences (selectivity).

Based on our experience [37–39] some rules in the process of single-residue method development using coupled-column RPLC for environmental samples should also be applied to urine samples:

(1) The clean-up volume, which is the volume of mobile phase used on column C-1, should at least be twice the dead volume of that column; in practice this means that the capacity factor, k, should be greater than 1.

(2) The capacity factor of the analyte in the mobile phases should not exceed 10, in order to achieve short times of analysis and good sensitivity.

(3) The eluotropic strength of the first mobile phase (M-1) must never exceed that of the second mobile phase (M-2).

(4) The use of gradients based on pH changes in M-1 and M-2 in the case of acidic or basic analytes.

Being a compound for which the availability of a rapid screening method is of great importance, and having rather favourable RPLC–UV properties (see Fig. 2 and Table 1), clenbuterol was selected to test the SRM methodology for the set-up of an automated coupled-column RPLC–UV procedure for its determination in samples of urine.

Initially, 25% methanol in acidic buffer (see above) was selected as the mobile phase on C-1 (M-1). It provides a clean-up of about ten column volumes (V_0) and an elution volume of clenbuterol of about 500 μl. For the second mobile phase, M-2, 40% of methanol was chosen, which gives a considerable peak compression on C-2, favourable towards sensitivity. Fig. 3A, shows the chromatogram obtained with column switching for the direct injection of 150-μl urine samples. Although the heart-cutting procedure seems rather efficient, the analyte elutes on top of interferences, which certainly will make the detection of clenbuterol at low levels difficult. Based on the experiences that acidic interferences can be removed by liquid–liquid extraction with an organic solvent from the acidified sample [5], a mobile phase on C-2 was prepared, containing the same constituents as before but without formic acid (pH of about 7.5), which should remove acid interferences as unretained compounds. Therefore, a gradient based on a pH change was investigated by using, as M-2, a mobile phase without the addition of formic acid. The obtained considerable increase in selectivity is clearly demonstrated in Fig. 3B.

The next step was to increase the sample injection volume. Experiments indicated that volumes up to at least 1.5 ml did not affect the elution volume of clenbuterol on C-1. Selecting this urine sample volume to perform on-line injections, only a part of the analyte containing volume (150 μl, see Fig. 4) was chosen as a good compromise between sensitivity and selectivity. The percentage of modifier in both mobile phases was also slightly adjusted to decrease clean-up time on C-1 and to increase separation on C-2.

The obtained coupled-column RPLC–UV procedure was tested with the analysis of human urine spiked with clenbuterol at a level of 10 μg/l. Examples of RPLC–UV analyses of a blank and a spiked urine sample are given in Fig. 5A and B, respectively. The method showed a good repeatability at this level (R.S.D. = 9.1%, $n = 8$), and moreover the system appeared to be robust over a period of two weeks in which at least 50 samples of 1.5 ml of urine were processed. It must be mentioned that sometimes a small shift in retention (4–6 s) of clenbuterol in different spiked urine samples was observed, requiring a re-adjustment of column switching conditions. An additional buffering of the sample of urine prior to injection may solve this inconvenience, but has to be further investigated.

The availability of a real-life bovine urine

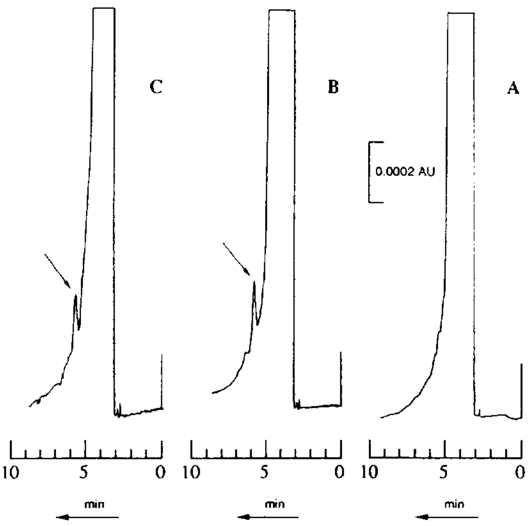

Fig. 5. Coupled-column RPLC–UV (245 nm) produced by the injection of 1.5 ml of (A) blank human urine, (B) blank human urine spiked with clenbuterol (10 μg/l) and (C) bovine urine sample containing clenbuterol. Clenbuterol is indicated by an arrow. Cleanup and transfer conditions as in Fig. 4; M-2 = 35% methanol in buffer without formic acid (pH about 7.5). After transfer of clenbuterol from C-1 to C-2, C-1 is rinsed with 95% MeOH (solvent B) for 5 min.

sample previously analysed by the Laboratory for Residue Analysis at the RIVM by GC–MS [27] and containing 6 μg/l of clenbuterol was a nice opportunity to test the procedure. As illustrated in Fig. 5C, clenbuterol was indeed found with the direct sampling method at the expected level (7.0 μg/l, duplicate measurement).

3.3. MRM approach

In the SRM approach discussed above the use of a very small transfer volume was essential to obtain selectivity using UV detection. For the simultaneous determination of all β-agonists having a wide polarity range (see Fig. 2), a large transfer volume will be necessary. Consequently, selectivity will be significantly lower excluding, in this case, the possibility of UV detection at low levels. Therefore, this study was directed to the development of suitable separation procedure to be used in next future in combination with TSP-MS detection.

To separate adequately the analytes a gradient elution will be necessary. Usually such a separation problem is solved with the selection of an appropriate linear gradient elution. In fact, using the already obtained information of Fig. 2 one should be able to estimate a suitable gradient elution profile. Two important aspects have been considered in this case: (i) to obtain sufficient retention of the firs eluting analyte (isoprenaline) and to perform clean-up the initial mobile phase must be at a low eluotropic strength (methanol < 5%) and (ii) the application of a rather steep gradient (after elution of isoprenaline) is desired to obtain small peak volumes (sensitivity). The result of a suitable linear gradient elution obtained with trial and error optimisation and conditions given in Fig. 6, is shown in Fig. 7A.

To our experience [36,37] step-gradient elution instead of linear gradient elution is preferable especially in conjunction with column-column LC where a step gradient (nearly) always occurs on the first column. Moreover, the availability of a fully automated optimisation procedure [40] for one- and/or two-step gradient elution profiles including relevant parameters (see below) made this type of elution attractive

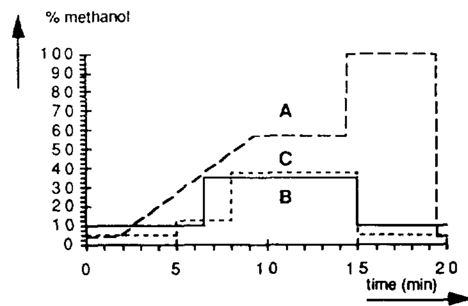

Fig. 6. Profiles of the adopted (A) linear, (B) 1-step and (C) 2-step-gradient elutions on a 3-μm Microspher C_{18} column (100 × 4.6 mm I.D.). The percentage of methanol in the mobile phase is plotted vs. time and starts with 4, 5 and 10% for A, B and C, respectively. Buffer composition as in Fig. 2.

for use. Therefore, as indicated in Fig. 2, step-gradient elution was investigated on its performance using the separation power of a 100 × 4.6 mm I.D. C_{18} column.

For the optimisation of the step-gradient elution conditions [36], the relevant parameters (i) minimal clean-up time, (ii) range eluotropic strength of mobile phases and (iii) the maximal time of analysis, were introduced into the computer program [40]. The calculated optimal conditions are presented in Fig. 6, and the corresponding chromatograms are shown in Fig. 7B en C. The comparison between calculated and experimental data is given in Table 2. The somewhat large differences for some analytes are probably caused by the use of a 100-mm column packed with material of a different batch than the 50-mm column used for the determination of the retention behaviour (Fig. 2).

As illustrated in Fig. 7, the three elution profiles provide an adequate separation of all analytes within 15 min. Concerning resolution, step gradient slightly favours linear gradient elution, while as regards the possibility to perform clean-up linear and two-step gradient elution are more suitable than one-step gradient elution. Owing to the possible negative effects of temporary very steep variations in the percentage of organic modifier in the mobile phase on the performance of the TSP interface, a linear gradient was initially selected for further experimental work.

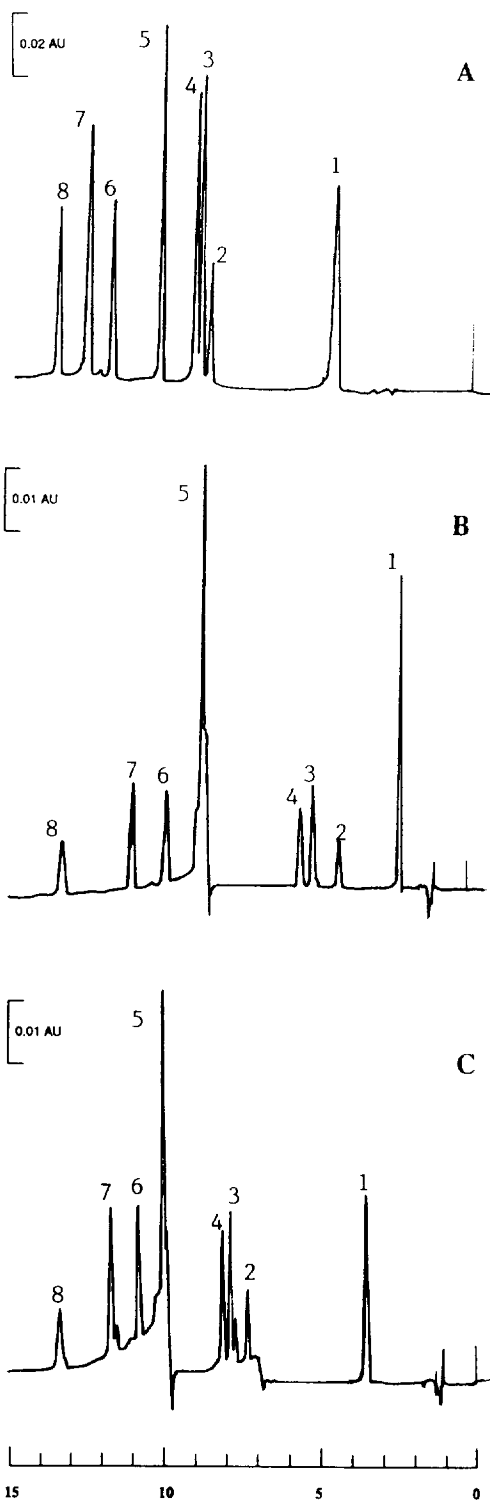

Fig. 7. RPLC–UV (285 nm) of the optimised separations obtained on a 100 × 4.6 mm, 3-μm, C_{18} column for the mixture of β-agonists with (A) linear, (B) 1-step and (C) 2-step gradient elution. Sample injection volume, 50 μl containing 1.5 μg (A) or 0.5 μg (B, C) of each analyte. Elution conditions as in Fig. 6. Peaks: 1 = isoprenaline; 2 = cimaterol; 3 = terbutaline; 4 = salbutamol; 5 = fenoterol; 6 = ractopamine; 7 = clenbuterol; 8 = mabuterol.

The coupled-column RPLC system with two 50-mm columns and the selected elution conditions (see Fig. 6 and 7A) was tested for the direct injection of urine samples. Based on preliminary RPLC–TSP-MS experiments carried out at Pavia, an injection volume of 150 μl of urine sample was selected to obtain an expected sufficient sensitivity. Fig. 8 presents a chromatogram produced by the established procedure. The selected clean-up time of 1.8 min (approx. 3 times V_0) was determined by the elution time of the first analyte isoprenaline on C-1. Although UV detection reveals the presence of many coeluting interferences, it is expected that TSP-MS will provide sufficient selectivity. Unfortunately selectivity could not be improved by using a buffer of higher pH on C-2 (see Section 3.2).

The robustness of the coupled-column procedure using acid mobile phases on both columns was tested by the injection of more than 50 urine samples, checking the performance of the columns by standard injections in between. It appeared that both retention and peak shape of the analytes were not affected, indicating that this approach is viable for the on-line determination of the β-agonists in urine samples in combination with TSP-MS.

It must be mentioned that an hydrolysis step prior to extraction is added in some published screening methods for β-agonists to include in the analysis also the fraction excreted in urine in the conjugated form (as glucuronates and/or sulphates). This fraction, though negligible for clenbuterol-like compounds, can be particularly significant for β-agonists with hydroxy groups on the aromatic ring(s) [26,29]. Although examples of notable urinary excretion of the unmodified drugs (up to 20% of total excretion) are available in the literature for terbutaline in man [18,43]

Table 2
Comparison of calculated (Calc.) and experimental (Exp.) chromatographic data of optimised step-gradient elution profiles given in Fig. 6 and shown in Fig. 7B and C

Compound	t_R (min)		σ (min)[a]	
	Calc.	Exp.	Calc.	Exp.
One-step gradient (Fig. 7B)				
Isoprenaline	1.54	2.18	0.020	0.025
Cimaterol	3.97	4.08	0.050	0.040
Terbutaline	5.00	4.90	0.063	0.040
Salbutamol	6.75	5.31	0.085	0.060
Fenoterol	7.97	8.41	0.014	0.020
Ractopamine	8.59	9.45	0.022	0.045
Clenbuterol	10.46	10.14	0.046	0.045
Mabuterol	16.24	12.88	0.119	0.065
Two-step gradient (Fig. 7C)				
Isoprenaline	2.78	3.45	0.035	0.040
Cimaterol	7.28	7.20	0.031	0.030
Terbutaline	7.78	7.75	0.036	0.035
Salbutamol	8.57	7.98	0.046	0.035
Fenoterol	9.46	9.82	0.014	0.025
Ractopamine	9.80	10.67	0.018	0.040
Clenbuterol	10.99	11.55	0.034	0.045
Mabuterol	14.26	13.22	0.075	0.055

[a] σ = Peak volume at 0.6 of the peak height.

and for salbutamol in calves [13], hydrolysis can be undoubtedly effective to increase sensitivity at the lower concentrations of residues in urine. Therefore, the influence of an acidic or enzymatic hydrolysis step in the presented procedure has to be evaluated. The possibility of a direct analysis of the conjugated forms was not investigated owing to the unavailability of reference standards.

4. Conclusions

The two basic approaches used in coupled-column RPLC as applied in pesticide residue analysis appeared to be effective for the analysis of β-agonists in urine samples. The usefulness of the first approach, the rapid detection of a single analyte, was clearly demonstrated for clenbuterol in urine samples. Applying large volume injection, carefully adjusted clean-up and transfer volumes, and proper mobile phase conditions, coupled-column RPLC–UV allowed the detection of clenbuterol in urine samples at the μg/l level. The short time of analysis (approximately 10 min) and the observed robustness of the LC system makes this approach highly useful for screening purposes.

The second approach, focussed at the simultaneous screening of eight β-agonists with a wide range in RPLC retention, provided easily suitable conditions for the automated processing of urine samples with coupled-column RPLC involving on-line clean-up and separation of the compounds within a time of analysis of less than 20 min. The processing of a large number of urine samples without any change in efficiency of the chromatographic system indicates that coupling of coupled-column RPLC to TSP-MS is a viable approach for the direct analysis of eight β-agonists in urine.

The applicability of the developed coupled-

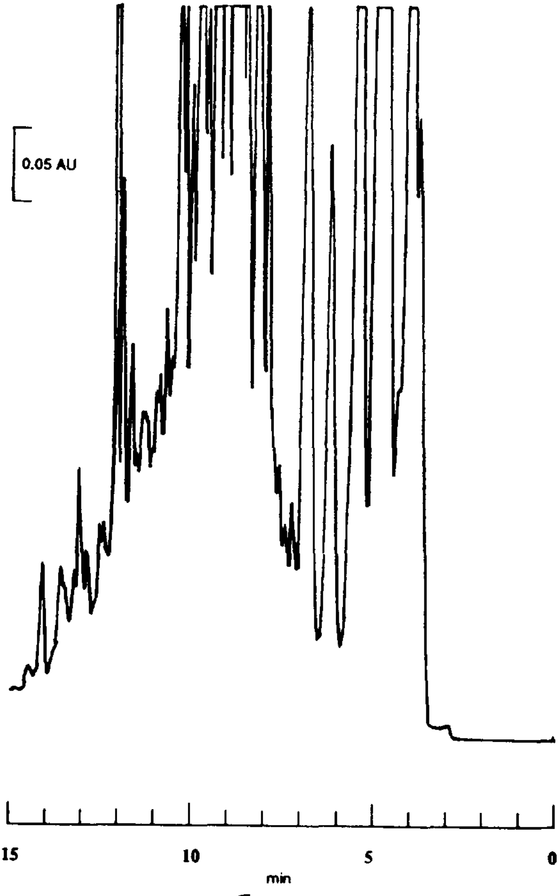

Fig. 8. Coupled-column RPLC–UV (285 nm) obtained for the injection of 150 μl of blank human urine. Clean-up time, 1.8 min; transfer time 13.2 min. For further explanation see text.

column procedures in combination with TSP-MS will be a matter of future research.

Acknowledgements

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