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Determination of β_2-receptor agonists in bovine urine and liver by gas chromatography–tandem mass spectrometry

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ABSTRACT

A highly specific and sensitive method for the simultaneous detection of seven β_2-receptor agonists in bovine liver homogenates and urine was developed. A 10-g amount of liver was homogenized and treated with Subtilisin A®. The resulting enzymatic digest was extracted with *tert.*-butanol–ethyl acetate (3:7) and the crude extract was purified on a 6-ml Bakerbond® alumina neutral disposable extraction column. Subsequently, the hydrous eluate from the alumina column was buffered at pH 6 and loaded on top of a preconditioned 3-ml Bond-Elut Certify® column. Urine was buffered and loaded onto a 3-ml Certify column without pretreatment. The analytes were eluted with dichloromethane–isopropanol (8:2) containing 2% ammonia. The extract obtained was trimethylsilylated and analysed by gas chromatography–tandem mass spectrometry using multiple selected reaction monitoring. The limits of detection for the β_2-receptor agonists evaluated were between 0.5 and 5 ppb.

INTRODUCTION

β_2-Receptor agonists (βRAs) are frequently used in man and animals for the treatment of chronic, obstructive pulmonary disease and bronchospasm. Increased protein synthesis and lipolysis are well known side-effects, which make βRAs also very useful as growth-promoting agents in animal breeding [1]. Although clenbuterol (CLEN) was probably the first βRA to be used for this purpose, at least some of the analogues listed in Fig. 1 were found to be potent substitutes. Therefore, a reliable screening procedure which detects βRAs at the low ppb level is needed for efficient meat control.

Previously, most of the work done in this field has been focused on CLEN. CLEN was isolated from urine using columns containing a modified form of diatomaceous earth. The crude extract was further purified by solvent extraction and subsequently analysed by high-performance liquid chromatography (HPLC) with UV detection [2]. Addition of a post-column derivatization step enhanced the UV sensitivity and selectivity [3]. Together with high-performance thin-layer chromatography (HPTLC) as a confirmation method for positive samples, this method has proved to be suitable for the detection of CLEN residues in urine and tissues samples at the low ppb level [3]. However, owing to its outstanding selec-

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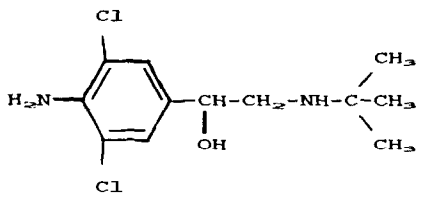

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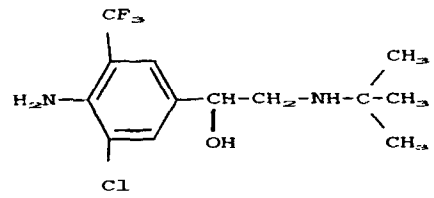

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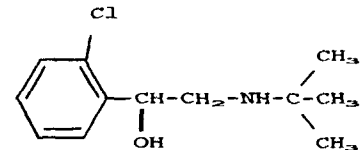

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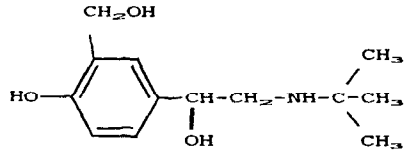

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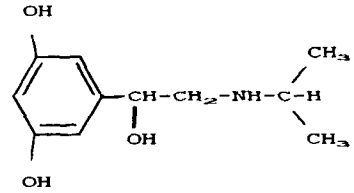

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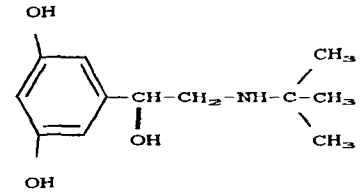

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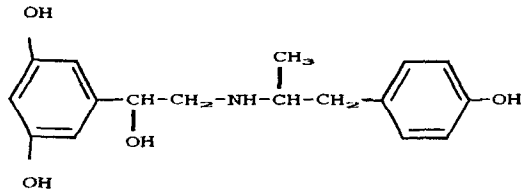
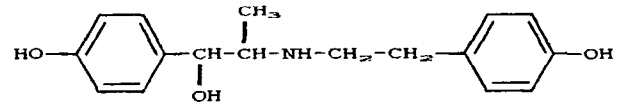


FENOTEROL



RITODRINE

Fig. 1. Structures of βRAs.

tivity and sensitivity, several laboratories prefer gas chromatography–mass spectrometry (GC–MS) [4–6] or liquid chromatography–mass spectrometry (LC–MS) [7]. More recently, the use of GC–MS in the negative ion chemical ionization mode allowed detection of CLEN in human plasma and urine at the femtomole level [8].

In our laboratory, the above-mentioned HPLC procedure [3] was adapted for GC–MS by adding a supplementary solvent extraction and trimethylsilylation step. This method was also found to be satisfactory for the determination of mabuterol (MABU) and tulobuterol (TULO) (data not shown). However, the phenolic analogues terbutaline (TERB), orciprenaline (ORCI) and salbutamol (SALB) were not detected.

Recently, a procedure has been described for the detection of SALB in urine from calves [9]. For pharmacokinetics studies, many methods, based on a wide range of extraction principles, have been published for the quantification of individual βRAs. These methods were predominantly based on HPLC with electrochemical or fluorescence detection and GC–MS with single ion monitoring. As the recoveries after solvent extraction were found to be low for the more hydrophilic phenolic βRAs, ion-pair extraction has been reported as a valuable alternative [10]. SALB has been extracted from plasma by solvent extraction [11], ion-exchange extraction [12], ion-pair extraction with di(2-ethylhexyl)phosphate [13,14], solid-phase extraction with octadecylsilica [9,15,16] or a combination of the latter two principles [17]. Extraction of TERB from plasma samples has been based on octadecylsilica [15,18–21], isolation with ion-exchange resins followed by extraction with *tert.*-butanol [12,22] or solvent extraction with ethyl acetate [23]. Also, plasma assays have been presented for TULO [24,25], CLEN [26,27], MABU [26], fenoterol (FENO) [15], ritodrine (RITO) [28,29] and reproterol [30], mainly based on solvent extraction. Only a few of the above-mentioned methods were designed for the simultaneous determination of two or three compounds [12,15].

In this study, a new extraction method was developed for the determination of seven βRAs in bovine liver homogenates and urine. GC–tandem MS (GC–MS–MS) was used for the simultaneous detection of the βRAs at the low ppb level.

EXPERIMENTAL

Chemicals and standards

The following reference compounds were kindly supplied by different companies: clenbuterol, orciprenaline sulphate, fenoterol (Boehringer Ingelheim, Brussels, Belgium), salbutamol (Glaxo, Greenford, U.K.), terbutaline sulphate (Astra-Nobelpharma, Brussels, Belgium), tulobuterol hydrochloride (UCB, Braine-l'Alleud, Belgium) and ritodrine hydrochloride (Duphar, Weesp, The Netherlands). Mabuterol was a generous gift from Dr. De Brabander (State University of Ghent, Ghent, Belgium).

All reagents and solvents were of analytical-reagent grade. Purified water was obtained from a consecutive Milli-RO and Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Macherey Nagel (Düren, Germany). Subtilisin A® was purchased from Novo Biolabs (Novo Industri, Copenhagen, Denmark). Bakerbond SPE® alumina neutral disposable extraction columns (6 ml) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Bond-Elut Certify® columns (3 ml) were obtained from Analytichem International (Harbor City, CA, U.S.A.).

Preparation of standard solutions and spiked samples

Stock solutions of standards (1 mg/ml) were prepared in methanol. A 100 μg/ml working solution was prepared by transferring 1.0 ml of each stock solution into a 10-ml volumetric flask and diluting to volume with purified water. This solution was used for the preparation of 500-ppb spiked samples. In a similar way, a 100 μg/ml working solution in chloroform–ethyl acetate (1:1) was prepared for recovery studies on alumina columns. A 100 ng/ml working solution was prepared by diluting 100 μl of the 100 μg/ml working solution to 100 ml with purified water.

In a similar way, 100 μg/ml and 100 ng/ml working solutions of RITO [internal standard (I.S.)] were prepared. Samples of 1 and 5 ppb were obtained by adding 100 or 500 μl (10 or 50 ng of each compound) of 100 ng/ml working solution to 10 ml of water, 10 ml of urine or 10 g of liver. Stock and working solutions were stored at 4°C. Spiked samples were freshly prepared.

Enzymatic digestion

A 10-g amount of calves' liver was weighed into the 50-ml stainless-steel chamber of an Omni-Mixer 17220 (Sorvall, Newton, CT, U.S.A.) and 10 ml of 0.2 *M* Tris buffer containing 0.1 *M* $CaCl_2$ (pH 8) were added. After homogenization at maximum speed for 2 min, the homogenate was treated with 10 mg of Subtilisin A by incubation for 1 h at 55°C with regular shaking. Subsequently, the enzymatic digest obtained was centrifuged for 10 min at 2800 *g*. Finally, the supernatant was saturated with sodium chloride and adjusted to pH 9.8 with 25% sodium hydroxide solution.

Solvent extraction and clean-up with alumina neutral extraction columns

The enzymatic digest of the sample was extracted twice with 8 ml of *tert.*-butanol–ethyl acetate (3:7) and the combined organic layers were evaporated to dryness in a rotating vacuum evaporation apparatus at 50°C. The residue was dissolved in 10 ml of chloroform–ethyl acetate (1:1) and dried over anhydrous magnesium sulphate. A 6-ml Bakerbond SPE alumina neutral disposable extraction column was conditioned with 10 ml of chloroform–ethyl acetate (1:1). Care was taken to prevent air from reaching the stationary phase during this step. The crude sample extract was then transferred to the top of the column at *ca.* 2

ml/min. The column was rinsed with 10 ml of acetonitrile and eluted with 5 ml of water. To the eluate, 2 ml of 0.1 *M* phosphate buffer (pH 6) were added.

Extraction with Bond-Elut Certify columns

A 3-ml Bond-Elut Certify disposable extraction column is activated with 2 ml of methanol followed by 2 ml of 0.1 *M* phosphate buffer (pH 6). Care was taken to prevent air from reaching the stationary phase during this step. The eluate from the alumina column was transferred to the top of the column at *ca.* 2 ml/min. The column was rinsed with 1 ml of acetic acid followed by 6 ml of methanol. Elution was carried out with dichloromethane–isopropanol (8:2) containing 2% ammonia. For recovery studies at the 1-ppb level, 300 μl of a 100 ng/ml internal standard working solution (30 ng of RITO) were added at this stage. For recovery studies at the 500-ppb level, 50 μl of 100 μg/ml internal standard working solution (5 μg of RITO) were added instead. The extract was carefully evaporated under a stream of nitrogen and derivatized with 25 μl of MSTFA overnight at 80°C. Finally, 1 μl was injected into the GC or GC–MS–MS system.

Gas chromatography

All GC measurements were performed on a Varian Model 3300 gas chromatograph equipped with an on-column capillary injector and a flame ionization detector. The column used was a WCOT RSil 300 fused-silica (d_f = 0.2 μm) column (30 m × 0.32 mm I.D.) (Alltech-RSL, Eke, Belgium). The temperature settings were as follows: injector, programmed from 60 to 250°C at 33°C/min; detector, 250°C; oven, programmed for 60 to 280°C at 8°C/min. Peak integration was performed on a Hewlett-Packard Model 9816 microcomputer with Nelson Model 4416 integration software.

Gas chromatography–tandem mass spectrometry

A Finnigan TSQ 70 GC–MS–MS system was used. The GC column used was a WCOT RSL 150BP fused-silica column (30 m × 0.32 mm I.D.) with a 0.25-μm film thickness (Alltech-RSL). The inlet pressure was 83 kPa. The temperature settings were as follows: injector, 280°C; transfer line, 280°C; oven, programmed for 80 to 230°C at 15°C/min and from 230 to 320°C at 30°C/min. A 1-μl volume was injected in the splitless mode.

The instrument was operated in the positive ion chemical ionization mode with isobutane as the reagent gas at a source pressure of 445 Pa. The electron energy was set at 70 eV and the ionization current was 200 μA. Multiple selected reaction monitoring was used to confirm the presence of the βRAs. At different time intervals, depending on the retention times of the analytes, the appropriate $[M + H]^+$ ions were selected as parent set masses in the first quadrupole. Collision-induced dissociation was achieved by using argon as collision gas at a collision cell pressure of 0.2 Pa. The applied collision energy varied between −30 and −50

TABLE I

GAS CHROMATOGRAPHIC DATA, MASS SPECTROMETRIC DATA AND LIMITS OF DETECTION (LOD) OF βRAs EVALUATED

βRA	Derivative	t_{Rabs} (min:s)	t_{Rrel}	*m/z*		Collision offset	LOD[b]	
				Parent ion, $[M+H]^+$	Daughter ions[a]		Liver	Urine
TULO	TMS1	8:15	0.580	300	154(100) 119(66) 91(24)	−50	0.5	<0.5
MABU	TMS2	10:42	0.752	455	217(100) 309(96) 289(62)	−45	0.5	<0.5
ORCI	TMS3	10:53	0.765	428	338(100) 296(36) 269(17)	−35	8	1
TERB	TMS3	11:01	0.774	442	296(100) 269(32)	−45	2	0.5
SALB	TMS3	11:30	0.808	456	220(100) 366(40)	−35	0.5	<0.5
CLEN	TMS2	11:57	0.839	421	275(100) 203(25) 331(15)	−30	0.5	<0.5
RITO (I.S.)	TMS3	14:14	1.000	504	193(100) 414(22)	−40	–	–
FENO	TMS4	14:33	1.023	592	207(100) 179(13)	−50	5	1

[a] Values in parentheses are relative abundances.

[b] Defined as the minimum concentrations (in ppb) detected in spiked samples with a signal-to-noise ratio >3.

eV. For each compound two or three specific daughter ions were monitored with a dwell time of 33–50 ms.

For positive identification, the relative abundances of the daughter ions generated from the parent ion, which was selected within the preset retention time windows, must fit the experimental values (Table I). These were obtained from three replicate injections of 200 pg/μl pure standard mixtures on the same day. Semi-quantitative results were obtained using the peak-area ratios of the most prominent daughter ions of each analyte and the internal standard RITO at *m/z* 193.

RESULTS AND DISCUSSION

Derivatization and gas chromatography

Relative retention times (t_{Rrel}) of eight trimethylsilylated βRAs on an RSL 150BP column are listed in Table I. MABU-TMS1 (t_{Rrel} 0.672) and CLEN-TMS1 (t_{Rrel} 0.783) were not detectable under the derivatization conditions used. A minor secondary peak was found for ORCI (ORCI-TMS4, t_{Rrel} 0.830) with a typical abundance of 5% in relation to ORCI-TMS3. To reduce derivatization problems, the conditions must be kept constant and good-quality columns with clean liners should be used. Moreover, a regular check for unwanted derivatives should be part of the regular quality control scheme, as MABU-TMS1 and CLEN-TMS1 were only observed when using older columns.

Extraction studies with 500-ppb spiked samples

Preliminary studies on extraction techniques were carried out by GC with flame ionization detection (FID) using water, blank urine and blank bovine liver homogenates spiked with 500 ppb of TULO, MABU, ORCI, TERB, SALB, CLEN and FENO. RITO was added before derivatization as an internal standard.

Extraction on Bond-Elut Certify columns. Bond-Elut Certify solid-phase extraction columns have been introduced recently for the extraction of drugs from

TABLE II

MEAN RECOVERIES (THREE REPLICATES) FROM WATER, URINE OR LIVER SPIKED WITH 500 ppb OF βRAs (GC–FID RESULTS)

Matrix	Steps included			Recovery (%)						
	1	2	3	TULO	MABU	CLEN	ORCI	TERB	SALB	FENO
Water	–	–	Certify	57	75	75	68	84	73	85
Urine	–	–	Certify	47	82	51	13	25	32	50
Water[a]	tBuOH[b]	–	Certify	15	78	87	3	7	16	16
Water[a]	C_2H_5 OAc	–	Certify	65	98	96	5	25	26	37
Water[a]	tBuOH–C_2H_5OAc (3:7)	–	Certify	43	98	89	13	53	62	34
$CHCl_3$–C_2H_5OAc[b] (1:1)	–	Alumina	Certify	28	88	81	33	66	61	35
Liver[a] homogenate	tBuOH	Alumina	Certify	?[c]	61	56	3	13	26	24
Liver[a] homogenate	tBuOH–C_2H_5OAc (3:7)	Alumina	Certify	25	85	60	3	20	35	9

[a] Saturated with NaCl.

[b] tBu = *tert.*-butyl; OAc = acetate.

[c] No result owing to interference.

urine prior to GC or GC–MS analysis. Depending on the mobile phase and pH conditions, the interaction mechanisms can be either hydrophobic or ionic. Using the standard procedure for the extraction of amphetamines, as proposed by the manufacturer [31], recoveries of βRAs from water were found to range between 57 and 85% (Table II). In contrast to results obtained after solvent extraction (ethyl acetate, *tert.*-butanol) and reversed solid-phase extraction (octadecylsilica columns), recoveries of the CLEN analogues and the more polar SALB analogues were nearly equal. As chemical reactions of TERB with carbonyl compounds have been reported [22], the amounts of ethyl acetate used were kept to the absolute minimum. Therefore, with the same results, the elution was carried out with dichloromethane–isopropanol (8:2) containing 2% ammonia. On analysing spiked urine, the recoveries were found to be lower, probably owing to matrix effects. Nevertheless, the very low background in the urinary chromatographic patterns clearly confirmed the superior selectivity of this new extraction method.

Solvent extraction from liver homogenates. Owing to the high ionic strength, direct extraction of liver homogenates with 3-ml Bond-Elut Certify solid-phase extraction columns was not successful. Even after a 100-fold dilution of the sample, the recoveries remained negligible. In contrast to ionic interactions, it is known that hydrophobic interactions are favoured by high concentrations of ions present. Therefore, a prepurification step based on solvent extraction was incorporated. The desalting effect was maximized by saturating the hydrophilic layer with sodium chloride. The extraction was carried out at pH 9.8, which was found [23] to be the optimum pH for solvent extraction of TERB. Recoveries of βRAs using ethyl acetate, *tert.*-butanol or *tert.*-butanol–ethyl acetate mixtures in combination with Bond-Elut Certify extraction were evaluated. The highes recoveries were obtained with *tert.*-butanol–ethyl acetate (3:7) as the extraction solvent (Table II). A higher *tert.*-butanol content resulted in dirty liver extracts with a high residual salt content. Moreover, the results obtained after *tert.*-butanol extraction were not reproducible. Using *tert.*-butanol–ethyl acetate (3:7) as the extraction solvent, no degradation of TERB was observed, but in view of earlier observations [22] the analytical conditions should be rigorously followed.

Clean-up with alumina columns. Combined extraction of liver homogenates with *tert.*-butanol–ethyl acetate (3:7) and Bond-Elut Certify was insufficient for adequate sample clean-up before GC–MS–MS analysis. After preliminary tests with different types of disposable extraction columns, alumina neutral columns were evaluated further for their possible use in a supplementary clean-up step. The most polar solvent, yet not polar enough to prevent adsorption of the analytes on a 6-ml Bakerbond SPE alumina neutral column was found to be chloroform–ethyl acetate (1:1). The recoveries obtained with acetonitrile as the washing solvent and water as eluting solvent are listed in Table II. The removal of residual water and *tert.*-butanol before application of the crude extract to the alumina column was essential as traces of water were found to desorb the analytes from

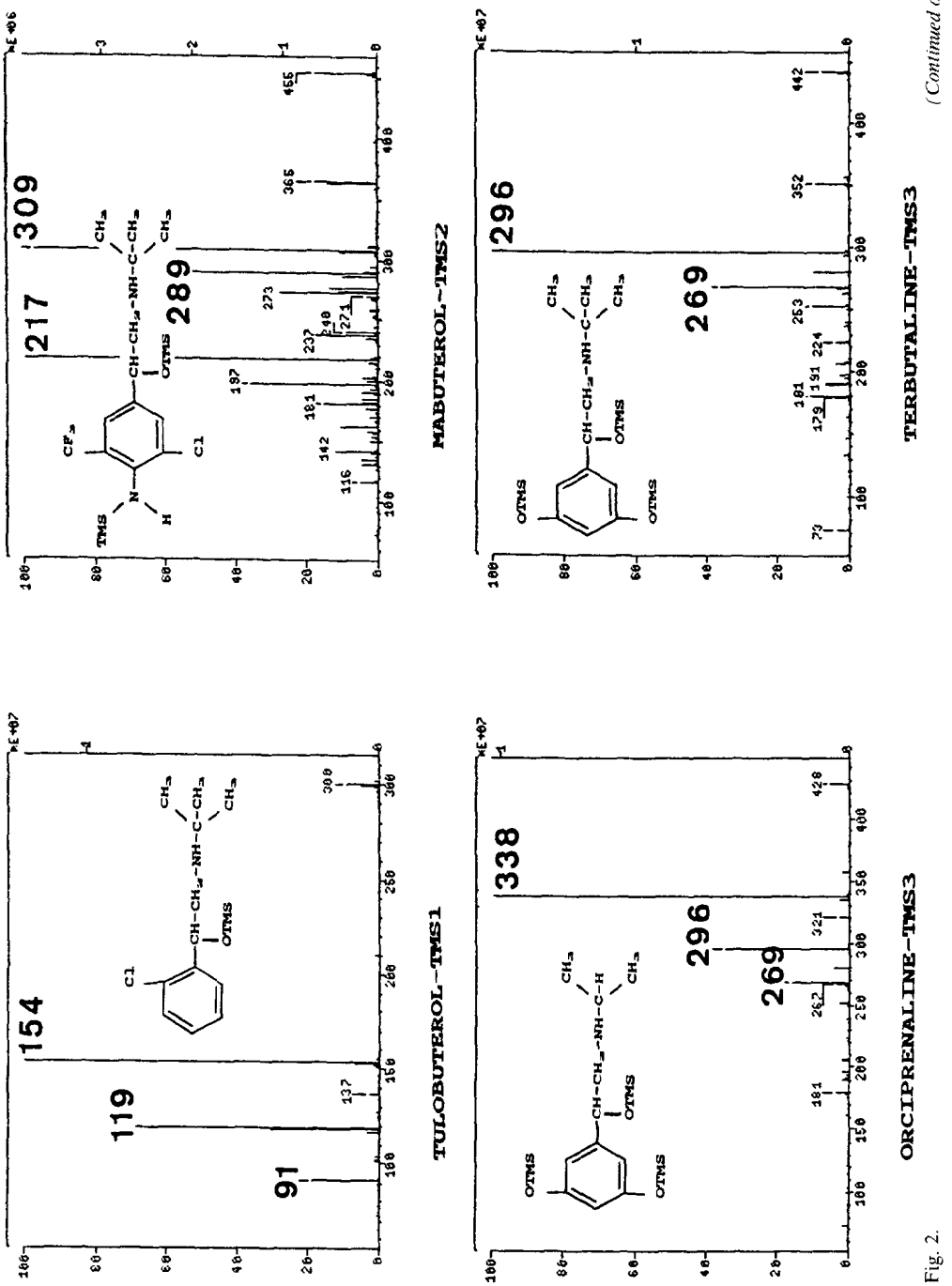


Fig. 2.

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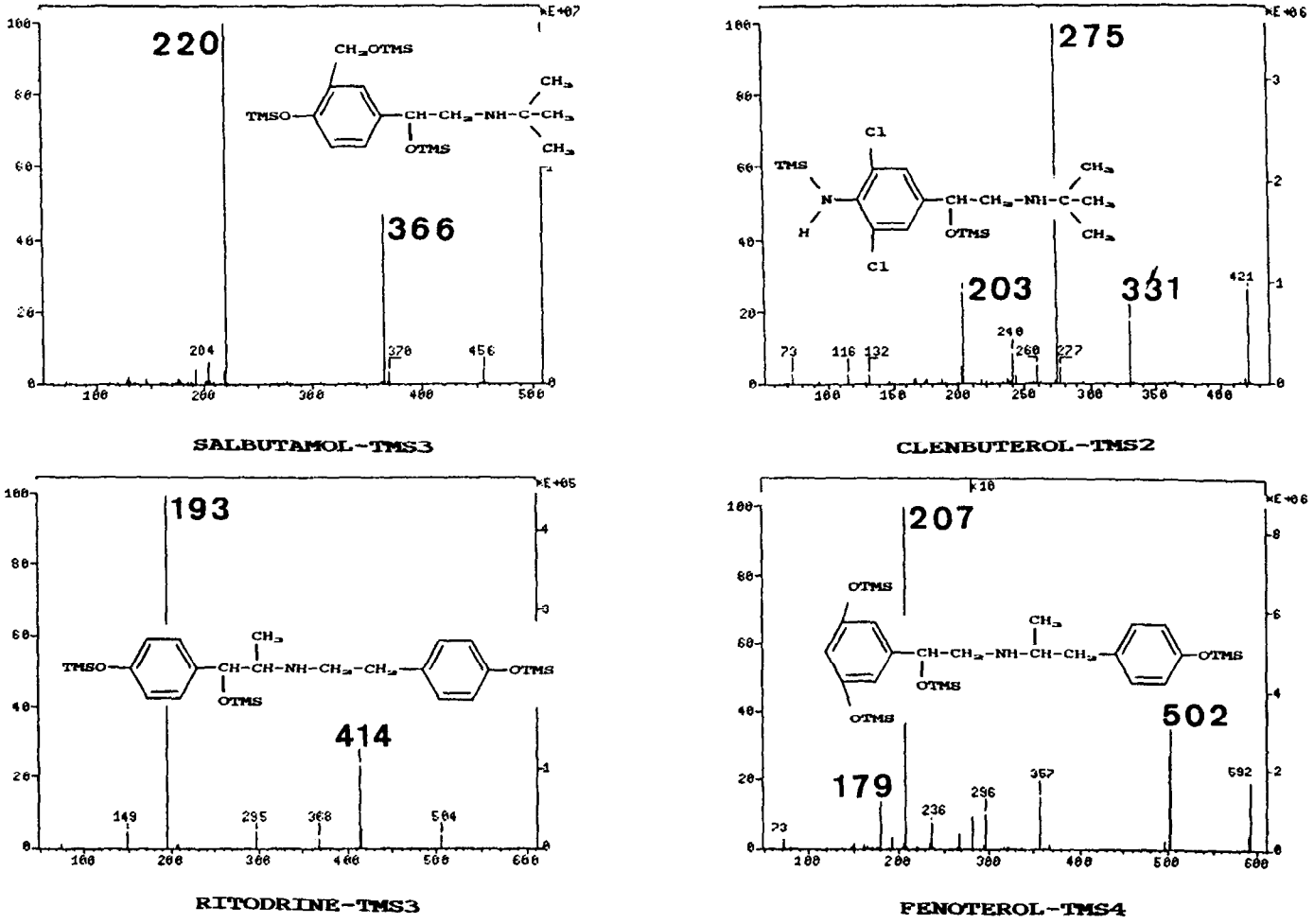

Fig. 2. Daughter-ion spectra observed after collision-induced fragmentation of the $[M+H]^+$ ion of trimethylsilylated βRAs.

the alumina column. The overall recoveries of the extraction method (Table II) were found to be acceptable for most of the compounds studied.

GC–MS and GC–MS–MS analysis

GC–MS–MS is a powerful new technique which has added nearly absolute selectivity to target compound analysis. Identification is based on (1) the reten-

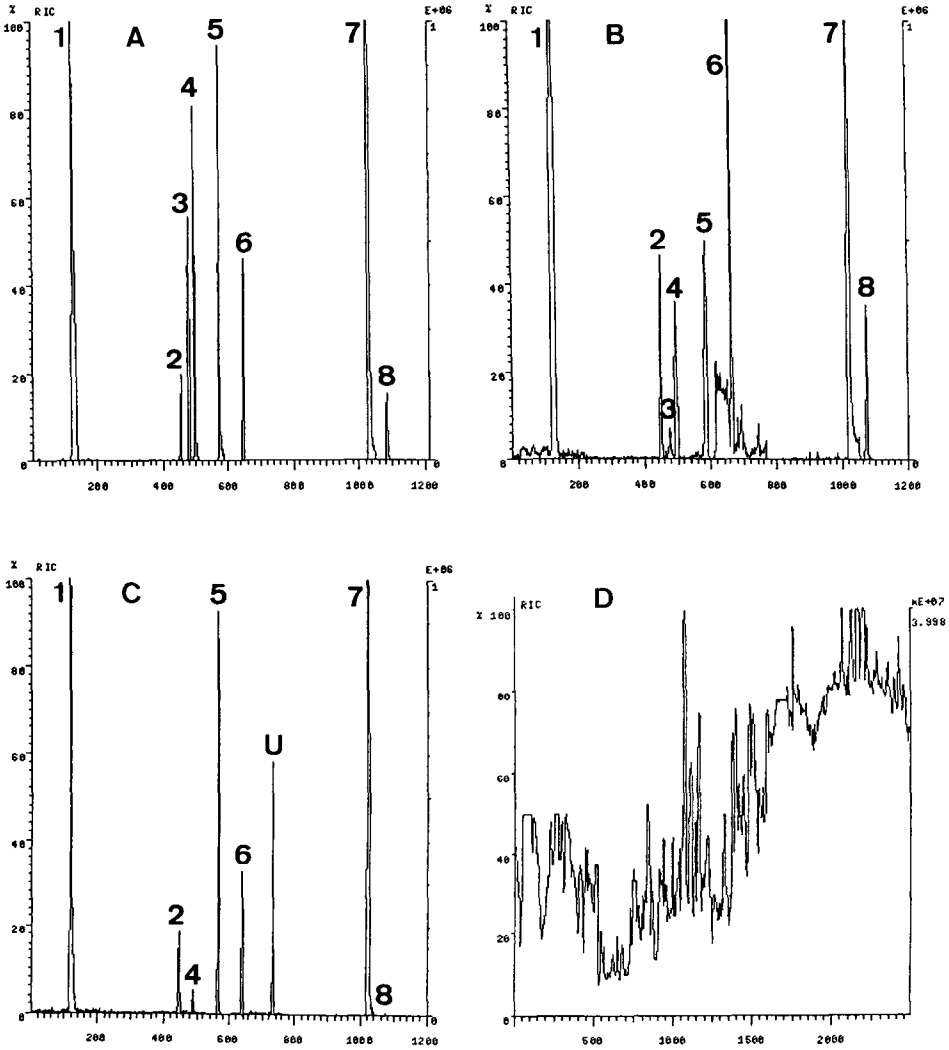

Fig. 3. Total ion current profiles obtained from (A) a 200 pg/μl standard mixture, (B) an extract of bovine urine spiked at 1 ppb, (C) an extract of bovine liver spiked at 2 ppb and (D) the same extract of bovine liver analysed by conventional GC–MS–SIM. Peaks: 1 = TULO-TMS1; 2 = MABU-TMS2; 3 = ORCI-TMS3; 4 = TERB-TMS3; 5 = SALB-TMS3; 6 = CLEN-TMS2; 7 = RITO-TMS3 (I.S.); 8 = FENO-TMS4; U = unknown.

tion time, (2) the presence of a typical $[M + H]^+$ ion (parent ion) and (3) the ratio of two or three typical fragments (daughter ions) generated by collision-induced dissociation of the corresponding $[M + H]^+$ ion. These ions were selected from the collision-induced fragmentation spectra of the trimethylsilylated βRAs, shown in Fig. 2. The total ion current obtained after injection of 1 μl of a mixture containing 200 pg/μl of each analyte is shown in Fig. 3. The gain in selectivity by using the GC–MS–MS method, compared with conventional GC–MS, is also illustrated and resulted in very clear background signals during the analysis of spiked samples. The limits of detection were considered to be acceptable for residue analysis. Also, it was found that when applied to urine, spiked at 1–5 ppb, solvent extraction and clean-up with 6-ml alumina neutral columns did not improve the purity of the extracts. However, extracts from urine samples directly extracted on 3-ml Bond-Elut Certify solid-phase extraction columns, were sufficiently clean for analysis by GC–MS–MS (Fig. 3). Moreover, the limits of detection were at least two or three times better in urine samples. Both ORCI and FENO, the limits of detection of which in spiked liver samples were 8 and 5 ppb, respectively, were easily detected at the 1 ppb level in spiked urine.

CONCLUSION

The three-step extraction procedure described in this paper allowed the detection of seven βRAs in liver and urine samples. Excellent selectivity and sensitivity were obtained by the use of tandem MS in the chemical ionization mode and multiple selected reaction monitoring. Further studies will be focused on studying the performance of the method for the detection of other βRAs.

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