Journal of Chromatography, 488 (1989) 357–367
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4583

IMPROVED SCREENING METHOD FOR BETA-BLOCKERS IN URINE USING SOLID-PHASE EXTRACTION AND CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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(First received September 28th, 1988; revised manuscript received November 15th, 1988)

SUMMARY

An improved screening method for beta-blockers in urine is proposed, involving enzymatic hydrolysis, solid-phase extraction and capillary gas chromatography–mass spectrometry. Several extraction methods for beta-blockers, such as conventional liquid–liquid and solid-phase extraction procedures, have been evaluated for at least eight beta-blockers. Additionally, the gas chromatographic properties and mass fragmentation of the trimethylsilyl-trifluoroacetyl, trifluoroacetyl and cyclic *n*-butylboronate derivatives of beta-blockers have been compared and evaluated with respect to their efficiency for screening urine. The resulting screening method proved to be a specific and sensitive procedure, enabling these analytes to be detected and identified up to 48 h after the administration of a dosage, usually encountered in doping cases.

INTRODUCTION

From January 1988, the Medical Commission of the International Olympic Committee (IOC) has forbidden the abuse of beta-blockers in sporting competitions. Low oral doses (5–100 mg) of these drugs were reported to be administered in order to decrease the heart-rate and/or muscular tremor in sports such as ski-jumping, archery, riflery and billiards. In 1987, 33 cases were reported in which a beta-blocker (most often propranolol and atenolol) had been used as a doping agent [1].

Unfortunately, typical systematic determinations of beta-blockers in urine with respect to extraction efficiency, sample clean-up and reproducibility are inadequate for doping analyses [2–4]. Within the last few years, solid-phase extraction (SPE) has been accepted as a convenient method in order to obtain clean extracts and optimum recoveries [5]. We evaluated systematically several extraction methods for at least eight beta-blockers in urine, including both conventional

0378-4347/89/$03.50

liquid–liquid extraction procedures and an SPE method, using C_2 bonded phases. In order to protect the bifunctional polar groups of the aminopropanol side-chain of beta-blockers, three different derivatization procedures, namely trimethylsilylation–trifluoroacetylation, trifluoroacetylation and cyclic boronation, were studied. Finally, several beta-blockers and their corresponding metabolites in human urine were identified and confirmed using capillary gas chromatography–mass spectrometry (GC–MS), which is highly accurate, specific and sensitive.

EXPERIMENTAL

Reagents

1-Pentanol (*n*-amyl alcohol), "zur Synthese", was purchased from Merck (Darmstadt, F.R.G.), *tert.*-butyl alcohol (purum) from Fluka (Buchs, Switzerland), methanol (Baker grade) and acetonitrile (HPLC grade) from J.T. Baker (Deventer, The Netherlands) and ethyl acetate (Uvasol) from Merck. All other routine chemicals were of analytical-reagent grade from Merck. N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and N-methylbistrifluoroacetamide (MBTFA) were purchased from Macherey, Nagel & Co. (Düren, F.R.G.), trifluoroacetic anhydride (TFAA) from Pierce (Rockford, IL, U.S.A.) and 1-butaneboronic acid from Aldrich (Milwaukee, WI, U.S.A.).

All beta-blockers were kindly supplied by their manufacturers: acebutolol hydrochloride, alprenolol hydrochloride, anhydrous atenolol (ICI Holland, Rotterdam, The Netherlands); labetalol hydrochloride (Schering, West Berlin, F.R.G.); metoprolol tartrate (Hässle, Mölndal, Sweden); nadolol (Squibb, Rijswijk, The Netherlands); oxprenolol hydrochloride, sotalol (Bristol-Myers, Weesp, The Netherlands); pindolol (Sandoz, Uden, The Netherlands); penbutolol sulphate (Hoechst Holland, Amsterdam, The Netherlands); bufuralol hydrochloride (Hoffmann La Roche, Mijdrecht, The Netherlands); betaxolol hydrochloride (Lorex Pharmaceutics, Amsterdam, The Netherlands); bisoprolol fumarate (Merck Nederland, Amsterdam, The Netherlands); and propranolol hydrochloride (ICI Holland).

Acetate buffer was prepared by mixing 25 ml of 1 *M* hydrochloric acid with 50 ml of 1 *M* sodium acetate and dilution with distilled water to 250 ml. The pH was adjusted to 5.2. Boronic acid–borax buffer (pH 9.0) was prepared by mixing 18.5 ml of 0.2 *M* boronic acid solution with 81.5 ml of 0.05 *M* borax solution. Acidified methanol was prepared by mixing 300 mg of acetic acid with 50 ml of methanol.

Standards

Separate stock solutions of the respective beta-blockers were prepared either in acetonitrile (bufuralol), methanol (pindolol, nadolol) or distilled water (all others) at a concentration of 0.2 g/l. These solutions were stored at 4 °C for not longer than 1 month and used to spike blank human urine.

Equipment

Bond-Elut C_2, C_{18} and CN solid-phase columns were purchased from Analytichem (Harbor City, CA, U.S.A.). The vacuum manifold was purchased from J.T.

Baker. The derivatized samples were analysed using a Finnigan-MAT ion trap detector (ITD) 800 with software revision 3.0. The AGC program with variable ionization time was used. The ionization time was set at 25 000 μs at low background (GC at 50°C) while tuning was performed using the automatic program. The multiplier was set at 150 V higher than the 10^5 gain test indicated. The ITD 800 was scanned from mass 50 to 500 in 1 s using the electron-impact (EI) mode.

The gas chromatograph was a Carlo Erba HRGC 5160 MEGA with on-column injector. A J&W Durabond 1 (30 m×0.25 mm I.D.) capillary column with a film thickness of 25 μm was used. The temperature programme was as follows: 1 min isothermal at 90°C, 40°C/min to 260°C, followed by an isothermal period of 15 min at 260°C. The sample volume injected was 1 μl. Only disposable glassware was used.

Extraction methods

Extraction A [2]. A 5.0-ml volume of urine was mixed with 1 ml of 1 *M* acetate buffer (pH 5.2) in a 10-ml glass tube, 5 ml of distilled diethyl ether were added and the sample was mechanically shaken for 20 min. After centrifugation for 10 min at 2000 *g*, the organic layer was discarded. The aqueous layer was adjusted to pH 9.6 with sodium carbonate–potassium carbonate (2:1, w/w). Subsequently, 1 ml of *tert.*-butyl alcohol, 5 ml of distilled diethyl ether and 3 g of anhydrous sodium sulphate were added. The mixture was shaken mechanically for 20 min and centrifuged for 10 min at 2000 *g*. Finally, the organic phase was removed and evaporated to dryness under a gentle stream of nitrogen at 30–35°C for 5 min and at 55–60°C until dry.

Extraction B. A 5.0-ml volume of urine was mixed with 1 ml of 1 *M* acetate buffer (pH 5.2) in a 10-ml glass tube, 5 ml of distilled diethyl ether were added and the sample was mechanically shaken for 20 min. After centrifugation for 10 min at 2000 *g*, the organic layer was discarded. The aqueous layer was adjusted to pH 9.6 with sodium carbonate–potassium carbonate (2:1, w/w). Subsequently, 5 ml of chloroform–1-pentanol (3:1, v/v) were added. The sample was shaken mechanically for 20 min and centrifuged for 10 min at 2000 *g*. Finally, the organic phase was removed and evaporated to dryness under a stream of nitrogen at 55–60°C.

Extraction C. The Bond-Elut columns were positioned in the respective Luer fittings of the vacuum manifold. A vacuum of 25–50 cmHg was applied. The columns were conditioned by eluting twice with 1 ml of methanol, once with 1 ml of distilled water and once with 1 ml of buffer (pH 9.0). The columns were prevented from running dry. An aliquot of 1 ml of urine or hydrolysed urine, containing a beta-blocker and the internal standard, was added to each column and gently sucked through. The columns were washed twice with 1 ml of distilled water and once with 500 μl of acetonitrile. Elution of the analytes was performed either with 500 μl of acidified methanol solution or with 500 μl of methanol. The eluates were evaporated to dryness under a gentle stream of nitrogen at 55–60°C.

Derivatization methods

Derivatization A [2]. A 100-μl volume of MSTFA was added to the dried residue and vortexed for 5 s and the tube was heated at 60°C for 5 min. Subsequently, 30 μl of MBTFA were added and the tube was again heated at 60°C for 5 min. A 1-μl volume of the cooled mixture was injected into the gas chromatograph.

Derivatization B. A 50-μl volume of TFAA–ethyl acetate (2:1, v/v) was added to the dried residue and vortexed for 5 s and the tube was heated at 60°C for 40 min. Subsequently, the mixture was evaporated to dryness under a stream of nitrogen at room temperature and the residue dissolved in 50 μl of ethyl acetate. After vortexing for 5 s, 1 μl of this mixture was injected into the gas chromatograph.

Derivatization C. A solution of 1-butaneboronic acid in ethyl acetate (0.1 g/l) was dried over anhydrous sodium sulphate prior to use; 50 μl of this solution were added to the dried residue. After vortexing for 5 s, 1 μl of this mixture was injected into the gas chromatograph.

Recovery experiments

A 100-μl volume of the stock solution, containing one or more beta-blockers (0.2 g/l), was mixed with 50 μl of the internal standard solution (bufuralol in acetonitrile, 0.2 g/l) and evaporated under a gentle stream of nitrogen at 55–60°C. In the meantime, 100 μl of the same stock solution were mixed with 1.00 ml (method C) or 5.00 ml (methods A and B) of blank human urine and subsequently extracted. Then, 50 μl of the internal standard solution were added to the extract, which was again evaporated under a gentle stream of nitrogen at 55–60°C. Finally, the dried residues were derivatized according to derivatization procedure A, except for atenolol, which was derivatized according to procedure B. All experiments were done in triplicate. The recoveries of the extractions were determined by comparing peak-area and peak-height ratios of the base peaks of the analyte with the internal standard before and after extraction.

Human sampling

Young, healthy volunteers ingested one tablet of the current dosage form of the respective commercially available beta-blockers: metoprolol tartrate (100 mg), atenolol (50 mg), propranolol·HCl (10 mg), sotalol·HCl (80 mg), penbutolol sulphate (50 mg) and labetolol·HCl (100 mg). Their urine was collected at 8, 24 and 48 h and 1 ml of the hydrolysed and 1 ml of the unhydrolysed urine were extracted according to method C and subsequently derivatized according to methods A, B and C (8-h samples) or method A (all other samples). Bufuralol (0.2 g/l in water) was used as the internal standard. Prior to analysis, the urine samples were stored at −20°C in the dark.

Hydrolysis

A 10-μl volume of a β-glucuronidase–arylsulphatase mixture from *Helix pomatia* (Serva, Heidelberg, F.R.G.) was added to 1.00 ml of urine sample and the samples were incubated at 37°C overnight.

RESULTS AND DISCUSSION

In preliminary studies using propranolol and oxprenolol as model drugs, we first optimized separately the SPE procedure. Although different bonded phases have been described for extracting a beta-blocker and/or its metabolite(s) from a biological matrix [6–12], we found high recoveries (70–90%) for a large number of beta-blockers, using C_2 bonded phases and acidified methanol as the eluent (Table I). Lower recoveries were obtained for the polar compound atenolol and also for labetolol, showing an aberrant structural feature. Using SPE, the extraction was more efficient than when conventional liquid–liquid extraction methods (A and B) for beta-blockers were used. In the latter instance, a loss of beta-blockers during the pre-extraction step at pH 5.2 can hardly be expected, as the pK_a of all beta-blockers except labetalol and sotalol is about 9.5. On the other hand, compounds may be adsorbed or form an inclusion complex with the sodium sulphate used in extraction method A and therefore be lost. The low recoveries of atenolol using method A or B may result from its high polarity.

Using MSTFA–MBTFA (method A) or TFAA (method B) derivatization procedures, the sensitivity and peak shape were generally better than when cyclic boronates (method C) were formed. However, a decrease in the resolving power of the capillary was observed if underivatized samples were analysed following the analysis of samples that had been derivatized using the MSTFA–MBTFA or cyclic boronation procedure. This undesirable side-effect was more significant using the cyclic boronation procedure and absent when TFA derivatives were formed. Using TFAA as the derivatizing reagent, side-product formation was ev-

TABLE I

RECOVERIES OF EXTRACTIONS OF BETA-BLOCKERS FROM URINE

A = Diethyl ether–*n*-butanol; B = chloroform–*n*-pentanol; C = SPE, elution with acidified methanol; C* = SPE, elution with methanol.

Drug	Recovery (mean ± S.D., $n=3$) (%)			
	A	B	C	C*
Acebutolol	–	–	80 ± 14	–
Alprenolol	20 ± 5	2 ± 0.4	90 ± 5	13 ± 1
Atenolol	2 ± 1	2 ± 0.6	59 ± 3	28 ± 4
Betaxolol	–	–	73 ± 3	–
Bisoprolol	–	–	79 ± 2	–
Bufuralol	–	–	97 ± 1	–
Labetalol	–	–	25 ± 6	–
Metoprolol	24 ± 3	9 ± 0.3	77 ± 2	19 ± 2
Nadolol	13 ± 1	27 ± 2	80 ± 2	42 ± 3
Oxprenolol	32 ± 10	34 ± 11	68 ± 12	40 ± 13
Penbutolol	29 ± 3	12 ± 2	73 ± 4	25 ± 7
Pindolol	43 ± 7	40 ± 4	86 ± 14	39 ± 4
Propranolol	25 ± 7	32 ± 5	87 ± 6	50 ± 12
Sotalol	–	–	74 ± 8	–

ident, which is disadvantageous for a screening method (Table II). Using the preferred MSTFA–MBTFA procedure, additional information about the metabolites could be obtained (Table III). All derivatives were stable for 2–3 days on storage at −20°C in the dark. The TMS–TFA derivatives were liable to hydrolysis owing to the presence of traces of acetic acid from the eluting solvent in the extract. In order to remove the acetic acid adequately, the respective extracts must be dried most carefully.

In Tables IV, V and VI the EI mass spectra of the TMS–TFA, TFA and cyclic boronate derivatives of the beta-blockers and metabolites are listed. For the TMS–

TABLE II

GAS CHROMATOGRAPHIC PROPERTIES OF DERIVATIVES A, B AND C OF BETA-BLOCKERS

Drug	Relative retention time[a] (min)		
	A	B	C
Bufuralol	1.00	1.00	1.00
Alprenolol	+0.46	−0.22	−1.14
Oxprenolol	+1.14	+0.02	−0.24
Penbutolol	+1.35	+1.25 (S)	+2.03
Metoprolol	+2.22	+0.46	+0.23
Pindolol	+2.38	+0.58	+2.26 (S)
	+5.05	+5.38	-
Propranolol	+3.15	+1.29	+2.00
Atenolol	+3.20 (T)	+1.08	+4.18
Sotalol	+3.30	+2.06 (S) (P)	+4.05 (T)
Bisoprolol	+3.40	−0.38	-
Betaxolol	+3.47	+1.54	-
Nadolol	+3.37	+0.10 (S)	+9.55
	+5.20	+0.46	+10.15
Labetalol	+9.15 (P)	+3.12	-
	+9.47	+3.12	-
Acebutolol	+15.00 (T) (P)	+8.34 (S) (P)	+2.24 (S)

[a]Retention times relative to internal standard bufuralol ($n=6$). T = tailing; S = side-product; P = poor GC properties (bad peak shape, low sensitivity).

TABLE III

SCREENING FOR BETA-BLOCKERS AND METABOLITES AFTER ORAL ADMINISTRATION OF ONE TABLET

P = Parent drug; M = metabolites and/or degradation products.

Time (h)	Hydrolysis	Atenolol	Metoprolol	Propranolol	Penbutolol	Labetalol	Sotalol
8	No	P	P,M	-	-	-	P,M
8	Yes	P	P,M	P,M	P,M	P,M	P,M
24	Yes	P	P,M	P,M	P,M	M	P,M
48	Yes	P	-	P,M	P,M	M	M

TABLE IV

EI MASS SPECTRA (m/z) OF TMS-TFA DERIVATIVES OF BETA-BLOCKERS (20–200 ng)

Relative abundances are given in parentheses.

Compound	Mol. mass	M+1	M−15	M−73	M−89	344	292	284	284−14	284−42	284−72	284−155	TMS	86	Others
Acebutolol	619							284(100)		242(7)		129(38)	73(33)		
4-Hydroxyalprenolol	505		490(2)		416(4)			284(100)		242(7)	212(2)	129(33)			
Alprenolol	417			344(2)	328(7)			284(100)	270(4)	242(4)		129(31)	73(36)		
Atenolol	433			360(2)				284(100)		242(7)		129(44)	73(74)		M(2)
Betaxolol	475		460(2)		386(4)			284(100)	270(2)	242(2)	212(2)	129(13)	73(9)		
Bisoprolol	493							284(100)	270(2)			129(13)	73(13)		332(7)
Bufuralol	333	334(67)												86(100)	M−86(4)/116(7)
Labetolol	550						292(100)								
Metoprolol	435		420(2)		346(7)			284(100)				129(26)	73(16)		235(2)
4-Hydroxymetoprolol	523							284(100)		242(2)		129(11)	73(36)		478(44)
Nadolol	525				436(4)									86(100)	
Oxprenolol	433		418(2)		344(2)			284(100)		242(3)		129(47)	73(44)		166(4)
Penbutolol	363	364(100)												86(27)	
Pindolol	512		497(2)		423(9)			284(100)			212(2)	129(37)	73(14)		
	416							284(100)				129(20)	73(20)		
Propranolol	427		412(2)		338(2)			284(100)		242(13)	212(3)	129(100)	73(42)		169(9)/115(30)
Sotalol	536					344(100)									272(2)

TABLE V

EI MASS SPECTRA (m/z) OF TFA DERIVATIVES OF BETA-BLOCKERS (20–200 ng)

Relative abundances are given in parentheses.

Compound	Mol. mass	M−113	308	308−42	308−156	126	86	69	Others
Alprenolol	441	328(7)	308(100)	266(29)					
Atenolol	458		308(100)	266(69)	152(11)				327(38)/132(7)
Betaxolol	499		308(100)	266(62)	152(10)	126(4)		69(7)	
Bisoprolol	517		308(84)	266(100)	152(33)	126(18)		69(75)	
Bufuralol	357						86(31)		262(100)
Metoprolol	459	346(9)	308(100)	266(40)	152(3)				M(2)/428(21)
Oxprenolol	457	344(44)	308(100)	266(31)	152(2)				M+1(3)
Penbutolol	387			266(100)					360(13)/162(13)
Pindolol	536	423(3)	308(91)	266(100)	152(28)	126(7)		69(22)	
	440		308(96)	266(100)	152(33)	126(27)		69(29)	
Propranolol	451	338(3)	308(100)	266(50)	152(7)			69(6)	M(5)/115(13)

TABLE VI

EI MASS SPECTRA (m/z) OF CYCLIC BORONATE DERIVATIVES OF BETA-BLOCKERS

Relative abundances are given in parentheses.

Compound	Mol. mass	M+1	M−15	Others
Alprenolol	315	316(92)	300(100)	180(67); 258(12)
Atenolol	332	333(4)	317(100)	
Betaxolol	373	374(26)	358(100)	182(19); 198(7) 286(8)
Bisoprolol	391	392(4)	376(16)	272(100); 288(4) 166(20); 182(14) 198(8)
Bufuralol	327	328(50)	312(100)	228(31); 182(12)
Metoprolol	333	334(12)	318(100)	183(11)
Nadolol	441	442(4)	426(100)	
Oxprenolol	331	332(19)	316(100)	218(71) 100(100) 274(47) 232(47) 180(38) 126(20) 84(27)
Penbutolol	357	358(18)	342(100)	
Pindolol	314		299(11)	M(100) 166(82) 104(67) 181(38) 132(38) 118(58)
Propranolol	325	326(60)	310(50)	128(100) 166(49) 182(33) 152(27) 84(31)
Sotalol	338		323(100)	239(24)

TFA (or TFA) derivatives of beta-blockers (Tables IV and V), the mass spectra contain in most instances universal and specific base peaks at 284 a.m.u. (or 308 a.m.u.), indicating the isopropylaminopropanol side-chain (Fig. 1). Other important base peaks of TMS–TFA derivatives of beta-blockers are at 86 a.m.u. (*tert.*-butylaminopropanol derivatives), 292 a.m.u. (labetol) and 344 a.m.u. (sotalol). In some instances the presence of the $[M-15]^+$ and $[M-89]^+$ ions in the mass spectra of TMS–TFA derivatives resulting from losses of a methyl and an OTMS group, respectively, allow a positive identification of the compound. The same is true for the presence of $[M-113]^+$ ions in the mass spectrum of the TFA derivatives, indicating a loss of a CF_3COO group. Other characteristic fragments at high mass that are specific for a compound had low abundances. In both instances the fragments at lower mass resulted from degradation of the isopropylaminopropanol side-chain, which is in accordance with the literature [2, 13].

The mass spectra of the cyclic boronate derivatives of beta-blockers (Table VI) are highly specific, as the base peak is usually the $[M-15]^+$ ion, resulting from a loss of a methyl group. The fragments having high abundances generally contained the boron atom, which could easily be recognized from the isotopic patterns. In the lower mass range, the fragmentation could be explained by a

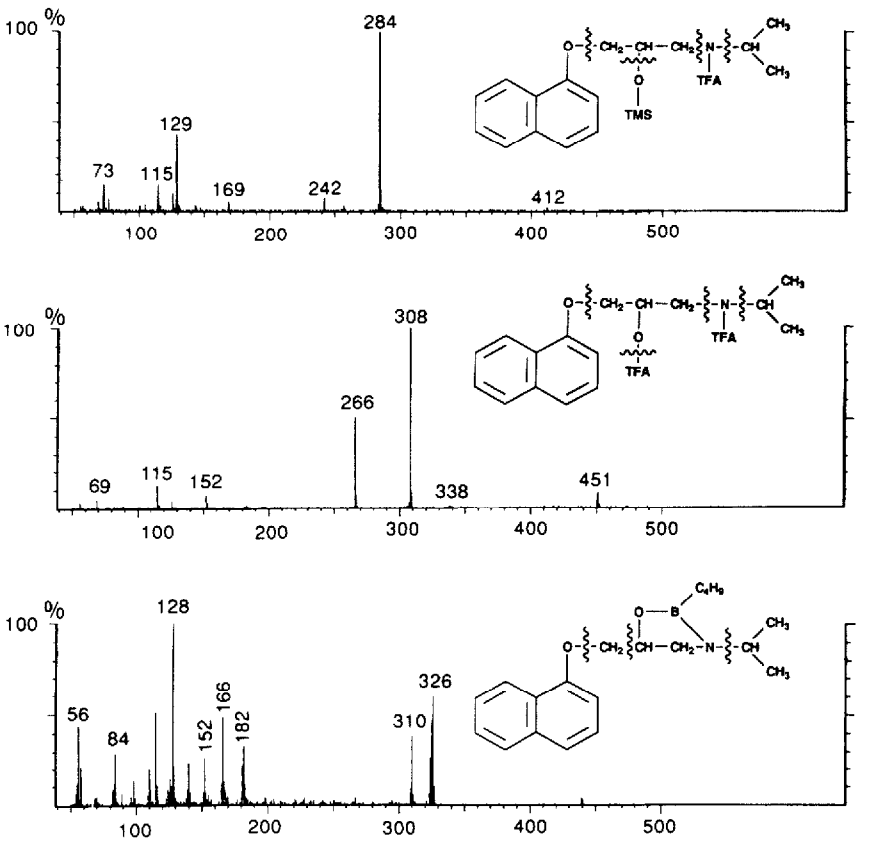

Fig. 1. EI mass spectra of the TMS–TFA, TFA and cyclic boronation derivatives of the model compound propranolol.

stepwise degradation of the *n*-butyl side-chain [14], in some instances after splitting off the aminopropanol side-chain. The occurrence of an $[M+1]^+$ ion in these mass spectra may be explained as a result of self-ionization in the ion trap mass detector, which has been well discussed recently [15]. For the screening of a structurally related group of compounds such as the beta-blockers using GC–MS, a universal and characteristic (base) peak is required. Therefore, TFA–TMS derivatives are to be recommended. Using the single ion monitoring mode, the sensitivity might be increased, but in doping cases proper confirmation with a full scan spectrum is essential. It is evident from our results that a mass range of 50–500 is sufficient to identify most beta-blockers adequately. Of course, more information may be gained if this mass range is widened, especially if metabolites are to be detected concomitantly.

Owing to the high sensitivity of the ion trap detector, beta-blockers and/or their metabolites could not only be detected but also identified from full scan data for urine up to at least 48 h after intake (Table III). Enzymatic hydrolysis was needed for most beta-blockers in order to obtain full information. The different peaks in the chromatograms may indicate the parent drug, metabolites and/or degradation products of metabolites. Further, chemical ionization information is needed to identify these metabolites and degradation products. These studies are currently in progress and will be discussed later.

CONCLUSION

We have proposed an improved screening method for beta-blockers and their metabolites in urine, involving enzymatic hydrolysis, a new and fast solid-phase extraction procedure using C_2 bonded phases and acidified methanol as the eluting agent, and an MSTFA–MBTFA derivatization prior to capillary GC–MS. The extraction efficiency of our SPE procedure is superior to that of conventional liquid–liquid extraction methods, such as those recommended by the IOC. The MSTFA–MBTFA derivatization procedure results in the formation of single derivatives with excellent GC properties and good stabilities. The EI mass spectra are specific and allow one to identify and confirm a beta-blocker and/or its corresponding metabolite(s) adequately. The high sensitivity of the ion trap detector permits the screening and identification of a beta-blocker and its metabolite(s) up to 48 h after an oral administration of the low doses that are usually encountered in doping cases.

ACKNOWLEDGEMENTS

We are greatly indebted to Mr. R.D. van Ooyen for skilfull technical assistance and to Mrs. M.V.A. van Wierst-Croese for assistance with the preparation of the manuscript.

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