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Comparative study of extraction methods for the GC and GC–MS screening of urine for beta-blocker abuse*

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Abstract: Comparison is made between conventional liquid–liquid extraction and solid-phase extraction techniques using Extrelut-1, Extrelut-3 and C18-RP cartridges respectively for the screening of several β-blockers in urine. Generally, using GC with nitrogen specific detection as the screening technique, liquid–liquid extraction and Extrelut-1 solid-phase extraction seem to be the methods of choice. However, when the screening and confirmation are performed by GC–MS, solid phase extractions with C18 or Extrelut-1 are valuable alternatives to conventional extraction. Using the different extraction techniques, detection limits and time periods during which the drugs are detectable in urine after oral administration of subtherapeutic amounts of several β-blockers are determined.

Keywords: *β-blockers; urine; doping analysis; GC GC–MS.*

Introduction

It is well known that β-adrenoceptor blocking drugs are of therapeutic value in the treatment of various cardiovascular disorders such as hypertension, angina pectoris and cardiac arrhytmia. However, these drugs can also be abused by athletes in order to reduce sympathetic activity in those cases where high psychic pressure could result in bad performances. As a consequence such drugs have recently been added to the list of forbidden substances recommended by the International Olympic Committee (I.O.C.).

Several methods are available for the analysis of β-adrenoceptor antagonists in biological fluids either alone or together with their metabolites. Studies on the screening of β-blockers are rather scarce, excepting the recent work of Martineau *et al.* [1]. In this study, β-blockers are detected by GC–MS in the selected ion monitoring mode (SIM) after derivatization by trifluoroacetic acid anhydride (TFAA). The monitoring of a single ion (m/z 266) can be used to screen about 80% of all β-blockers.

Although a GC–MS combination is a necessity in a doping analysis laboratory, the use

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of a mass spectrometer as a gas chromatographic detector for routine analysis is still an expensive technique. Furthermore, as screening of anabolic steroids and confirmation of positive samples for stimulants and narcotics also necessitates GC–MS equipment, mass spectrometric screening for β-blockers could result in a GC–MS work overload. Therefore screening by GC alone is worthy of consideration.

In the analysis of drugs in biofluids there is a major emphasis on isolation of the drugs of interest from the biological matrix. Appropriate sample clean-up will usually enable an analyst to obtain increased selectivity and sensitivity. Although techniques such as liquid–liquid extraction can provide the analyst with excellent recoveries, the methodology can be lengthy and tedious due to back-extractions and centrifugation. Moreover large volumes of solvents may be necessary. As a consequence solid phase extraction is gaining wider acceptance in doping analysis [2, 3] especially as the Extrelut system provides a means of achieving a rapid and precise extraction [4, 5]. Furthermore, the use of bonded silica phases is reported to be faster and more convenient than conventional liquid–liquid extraction. Moreover, the technique is amenable to automation [6]. Therefore, several extraction methods for the screening of β-blockers, both with GC–NPD and GC–MS, have been studied in this work. The respective detection times after oral administration are also determined.

Experimental

Reagents and chemicals

Trifluoroacetic acid anhydride (TFAA) was from Pierce (Rockford, U.S.A.). Diethylether, *n*-propanol and ethylacetate p.a. from Merck (Darmstadt, F.R.G.).

The following β-adrenergic blockers and metabolites were kindly supplied by their respective manufacturers: alprenolol, metoprolol and oxprenolol, Ciba–Geigy (Basle, Switzerland); nadolol, Squibb (Brussels, Belgium); pindolol, Sandoz (Basle, Switzerland); atenolol, propranolol and 4-hydroxy-propranolol, ICI (Destelbergen, Belgium); metoprolol metabolites and oxprenolol metabolites, AB Hässle (Mölndal, Sweden).

Helix Pomatia juice (SHP), β-glucuronidase 100,000 Fishman units ml^{-1} and sulfatase 1,000,000 Roy units ml^{-1}, IBF (Villeneuve, France).

Sodium acetate and acetic acid were used for the preparation of the 1.0 M acetate buffer pH 5.3. Sep-Pak C18 cartridges were obtained from Waters Assoc. (Milford, U.S.A.). Extrelut-1 and Extrelut-3 columns could be either purchased from Merck (Darmstadt, F.R.G.) or self prepared using equivalent amounts of Extrelut [5].

Gas chromatography

All chromatograms were generated in the split mode (split ratio 1:10) on a Varian (Walnut Creek, U.S.A.) 3400 gas chromatograph fitted with a 25 m × 0.22 mm i.d. fused silica CP Sil-5-CB column (Chrompack, Antwerpen, Belgium) with a film thickness of 0.11 μm. The GC was equipped with a nitrogen specific detector and interfaced with an IBDH data processor. Injector and detector temperatures were maintained at 250° and 300°C, respectively. For the analysis of a specific β-blocker, the oven temperature was programmed as follows: initial temperature 140°C, initial hold 1 min, temperature program rate 10°C min^{-1}, final temperature 280°C, final hold 5 min. Helium was used as the carrier gas at an inlet pressure of 1.1 bar. Detector make-up flow rate was 30 ml min^{-1}.

Gas chromatography–mass spectrometry

Mass spectra were acquired by means of a Hewlett–Packard (Avondale, PA, U.S.A.) 5993 mass spectrometer. The GC–MS system was fitted with the same fused silica capillary column as above. Sample injection was accomplished with an all-glass moving needle microsyringe (Chrompack, Antwerpen, Belgium). The column was directly coupled to the MS source. Ion monitoring data were acquired at an ionization potential of 70 eV. Injector, interface, source and analyzer temperatures were respectively 240°, 260°, 250° and 250°C. Helium was used as a carrier gas with an inlet pressure of 0.7 bar. The electron multiplier voltage was set at the autotune value.

Methods

Liquid–liquid extraction. The method of Martineau *et al.* [1] was slightly modified. To 5 ml urine was added 1 ml of 1 M sodium acetate buffer and 100 μl SHP. The samples were hydrolysed overnight (37°C). After cooling the hydrolysate was extracted (5 min) with 3 ml ethyl acetate by rolling.

After centrifugation at 700 **g**, the organic phase was discarded and the aqueous layer was made alkaline by adding 2 g of a $NaHCO_3/K_2CO_3$ (1:1) mixture and extracted (15 min) with 3 ml of diethylether–propanol (9:1 v/v). After centrifugation the organic layer was separated, dried over Na_2SO_4 and evaporated under nitrogen at 70°C.

Extrelut extraction. A recently published method [7] was used. (a) Extrelut-1. Urine (2 ml) was buffered with 0.5 ml of 1 M sodium acetate buffer and after addition of 50 μl SHP, hydrolysed overnight at 37°C. After cooling the hydrolysate was made alkaline by addition of 1 g $K_2CO_3/NaHCO_3$ 1:1, briefly centrifuged and 1 ml passed through the Extrelut-1 column. After 5 min the column was eluted with 3 ml of diethyl ether–propanol 9:1 and the eluate evaporated under nitrogen at 70°C; (b) Extrelut-3. The same method was used as for Extrelut-1 columns, excepting that 3 ml urine was used. Moreover, the total volume of hydrolysed urine was put on the Extrelut-3 column and elution performed with 9 ml of diethyl ether–propanol 9:1.

Sep-Pak extraction. To 3 ml of urine was added 0.5 ml of 1 M sodium acetate buffer and 50 μl SHP. Hydrolysis was performed at 37°C overnight. Sep-Pak C18 cartridges were activated before use by washing with 1 ml of methanol, followed by 5 ml of water. The hydrolysate was made alkaline by addition of 0.5 ml 0.5 M NaOH and 1 g of the $NaHCO_3/K_2CO_3$ mixture. After brief centrifugation at 700 **g** the urine hydrolysate was transferred to the cartridge with a syringe. The cartridge was washed with 5 ml water and sucked dry for at least 5 min. The β-blockers were recovered by passing 3 ml of the diethyl ether–propanol mixture through the cartridge with a syringe and evaporating the solvent under nitrogen (70°C).

Derivatization method. The residue was dissolved in 50 μl ethylacetate and treated with 50 μl TFAA. Derivatization was performed at 70°C for 20 min. Afterwards the reaction mixture was taken to dryness by a stream of nitrogen (70°C), the residue redissolved in 200 μl ethylacetate and 1 μl injected.

Detection time experiments. Several healthy persons participated in the excretion studies. Subtherapeutic amounts of the following β-blockers were given orally at 10 a.m.: Tenormin® (atenolol, 50 mg); Seloken® (metoprolol, 50 mg); Inderal® (propanolol,

20 mg); Visken® (pindolol, 5 mg); Trasicor® (oxprenolol, 40 mg) and Aptine® (alprenolol, 25 mg).

Urine was collected at 0 h and 1, 2, 3, 4, 6, 9, 12, 24 and 48 h after administration. Aliquots of each urine sample were analysed using the different extraction methods. The time period during which the β-blocker and/or its metabolites were detectable in urine (detection time) was evaluated using the different extraction methods.

Detection limit experiments. Methanolic solutions (100 μg ml^{-1}) were made for each β-blocker. Diluted stock solutions (10 and 1 μg ml^{-1}) were used to add different amounts (10, 5, 2 and 1 μg; 500, 200, 100, 50, 20 and 0 μg) of the β-blocker to screw capped tubes. After evaporation of the solvent, appropriate amounts of blank urine were added, followed by the buffer solution and SHP. The tubes were kept at 37°C overnight and analysed as described above. However, during the GC–MS analysis only the ions m/z = 308 and m/z = 266 were monitored.

Results and Discussion

Some β-blockers and their hydroxylated metabolites are partly or completely excreted as conjugates, therefore hydrolysis of the urine is necessary. As atenolol and pindolol are completely decomposed by acid hydrolysis at elevated temperatures [8] the release of the β-blockers from their conjugates by enzymatic hydrolysis was preferred.

The extraction procedure times for a hydrolysed urine were found to be approximately 5, 10 and 30 min respectively for the Extrelut-1, Sep-Pak and classical extraction methods. The longer analysis times for the C-18 system were due to the cartridge activation step and especially to the drying necessary before the elution of the β-blockers. Centrifugations, back-extraction and the long extraction time accounted for the higher process time observed with the classical extraction.

Although the recovery is a determinant factor in the evaluation of an extraction method, in doping analysis one is primarily interested in detection limits and especially in the time periods during which a drug can be detected after its administration (detection time).

The detection times for several beta-blockers and their metabolites after the oral administration of subtherapeutic amounts and screening by capillary gas chromatography are summarized in Table 1. Generally, the detection times using the classical extraction and 5 ml of urine are very good and these β-blockers could be detected during 1 day. The exception is pindolol where the low administered dose could account for the relatively shorter detection time.

Using Extrelut-1 columns and only 1 ml of hydrolysate the β-blocking agents were detectable at least till 6 h after their ingestion. The use of a larger volume of urine and Extrelut-3 columns apparently did not improve the results as a greater volume of urine gave rise to less clean extracts. Therefore this method has only been applied to propranolol and atenolol. Chromatograms obtained after Extrelut-1 extraction are shown in Fig. 1 illustrating the clean extracts and the detection of atenolol 24 h after its administration.

The GC detection times using Sep-Pak C18 cartridges and 3 ml of urine were equal or inferior to the classical method. However, as shown in Fig. 2 routine screening could be seriously hampered especially for β-blocker derivatives (oxprenolol, metoprolol, 4-OH alprenolol) with retention times in the range of 6–8 min.

Table 1
Detection times (h) of several β-blockers and their major metabolites after subtherapeutic administration (capillary gas chromatography with nitrogen specific detection)

	Liquid (5 ml)	Extrelut-1 (1 ml)	Extrelut-3 (3 ml)	Sep-Pak C18 (3 ml)
Propranolol	24	6	9	9
4-Hydroxypropranolol	24	—	—	9
Atenolol	24	24	48	48
Oxprenolol	24	6		12
4-Hydroxyoxprenolol	24	6		24
5-Hydroxyoxprenolol	24	6		24
2-Hydroxyoxprenolol	9	—		—
Pindolol	12	9		9
Metoprolol	24	6		12
α-Hydroxymetoprolol	24	6		24
Alprenolol	12	12		9
4-Hydroxyalprenolol	24	12		24

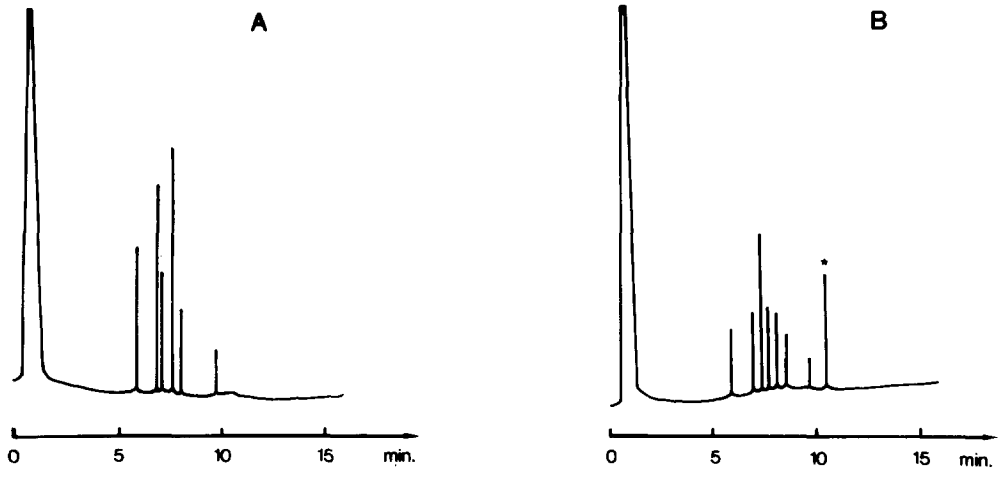

Figure 1
CGC of a blank urine extract (A) and urine obtained 24 h after atenolol administration (B) using Extrelut-1 extraction. Peak*: atenolol-di-TFA.

Generally, if screening of β-blockers is done by capillary GC, the following conclusions could be drawn depending on laboratory requirements and priorities. (i) If urine volume and/or analysis time is a determinant factor, the use of Extrelut-1 columns should be preferred; (ii) based on the quality of the gas chromatograms and consequently the occurrence of interfering substances, the Sep-Pak C18 cartridge method could create problems in the interpretation of the results; (iii) if a detection time of 24 h is necessary, the classical extraction method should be applied.

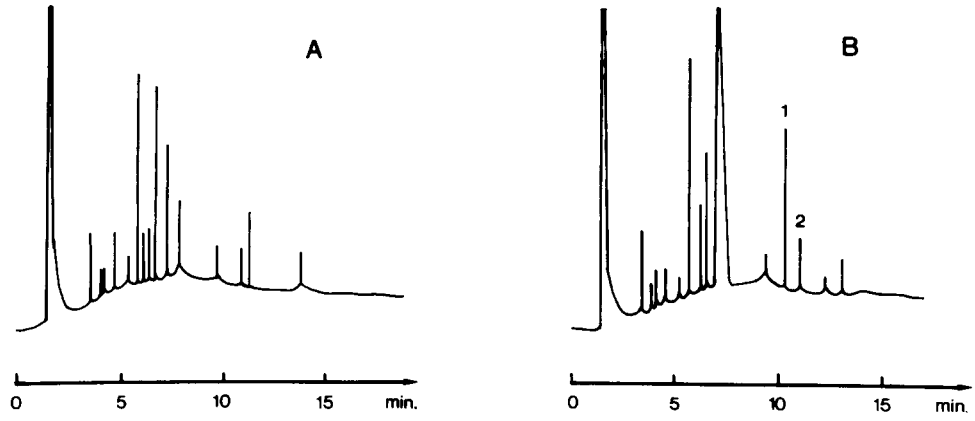

Figure 2
CGC of a blank urine extract (A) and urine 6 h after propranolol administration (B) obtained by Sep-Pak C18 extraction. Peak 1: propranolol-di-TFA. Peak 2: 4-hydroxypropanolol-tri-TFA.

Table 2
Detection times (h) of several β-blockers and their major metabolites after subtherapeutic administration (GC–MS, selected ion monitoring mode)

	Liquid (5 ml)	Extrelut-1 (1 ml)	Extrelut-3 (3 ml)	Sep-Pak C18 (3 ml)
Propranolol	24	24	24	48
4-Hydroxypropranolol	24	9	24	48
Atenolol	24	48	48	48
Oxprenolol	24	48		24
4-Hydroxyoxprenolol	24	48		24
5-Hydroxyoxprenolol	24	48		24
2-Hydroxyoxprenolol	24	—		9
Pindolol	24	24		24
Metoprolol	24	24		24
α-Hydroxymetoprolol	24	48		24
Alprenolol	12	12		12
4-Hydroxyalprenolol	24	24		24

When β-blockers having a 2-hydroxy-3-isopropylaminopropoxy side chain are derivatized with TFAA, their mass spectra give rise to abundant ions at m/z = 308, 266, 152 and 126; the base peak being either m/z = 308 or 266 [9]. Moreover, these ions are also common to the main metabolites as for most β-blockers the 2-hydroxy-3-isopropylaminopropoxy chain is not affected by metabolism. The detection times measured by the monitoring of these four ions are given in Table 2 and are obviously longer than for GC–NPD screening. Generally, these β-blockers and their major metabolites were detectable at least during one whole day and in some cases up to 48 h.

4-Hydroxypropranolol which was not detected by GC, after Extrelut-1 extraction is now detectable up to 9 h after the propranolol administration. Moreover, Extrelut-1

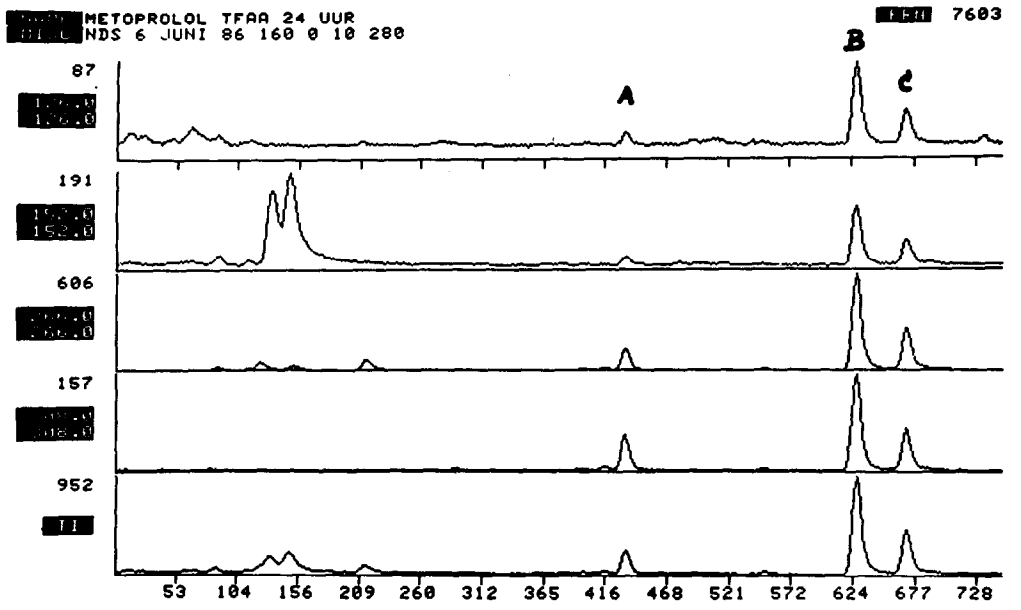

Figure 3
Mass fragmentogram and TIC of a urine extract 24 h after metoprolol administration (classical extraction). Peak A: metoprolol-di-TFA. Peak B, C: decomposition products of TFAA derivatized α-hydroxymetoprolol.

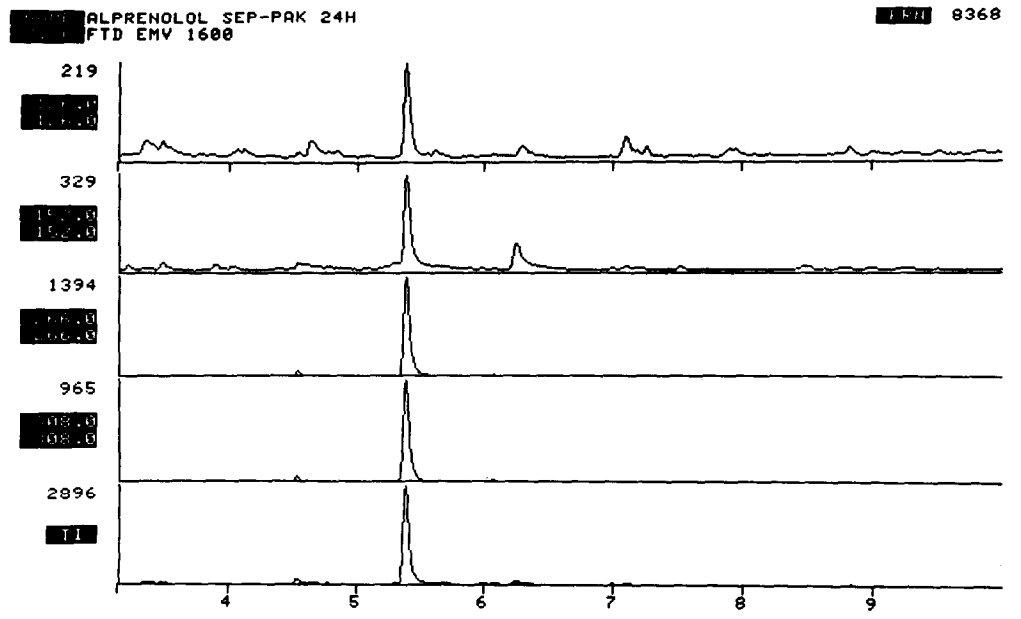

Figure 4
Mass fragmentogram and TIC of a urine extract 24 h after alprenolol administration (Sep-Pak extraction); peak with retention time 5.47 min corresponds to 4-hydroxyalprenolol-tri-TFA.

extraction using 1 ml urine surprisingly resulted in detection times that were equal or even longer than for the classical extraction method. The same could be said for Sep-Pak extractions.

A mass fragmentogram of metoprolol 24 h after its administration and using the classical extraction is given in Fig. 3. Apart from the parent compound two peaks resulting from the decomposition of α-hydroxymetoprolol during the GC analysis [10] were also found. The major metabolite of alprenolol has been identified as 4-hydroxyalprenolol [11], occurring in the urine largely in a conjugated form. The predominant peak in the mass fragmentogram of Fig. 4 refers to derivatized 4-hydroxyalprenolol found in a urine sample 24 h after the administration of alprenolol and using the Sep-Pak cartridge extraction system. Finally, the efficiency of Extrelut-1 column extraction for doping analysis of β-blockers is demonstrated in Fig. 5 where atenolol was easily detected in a 1 ml urine sample 48 h after its administration.

The detection limits for several β-blockers using the different extraction methods are given in Table 3. Taking into account the different volumes of urine, respectively 5, 1 and 3 ml for conventional, Extrelut and Sep-Pak extraction it is apparent that the Extrelut-1 procedure and GC–NPD generally give rise to relatively lower detection limits. Compared to other β-blockers, the detection limit for pindolol is obviously higher for all three methods. This could be due to the instability of the TFA-derivative of pindolol at higher temperatures [M. Donike, personal communication]. This higher detection limit and the relatively low administered dose could both account for the short period during which pindolol could be detected.

The detection limits using GC–MS and monitoring the ions m/z = 308 and 266 are sufficient to meet the requirements of doping analysis.

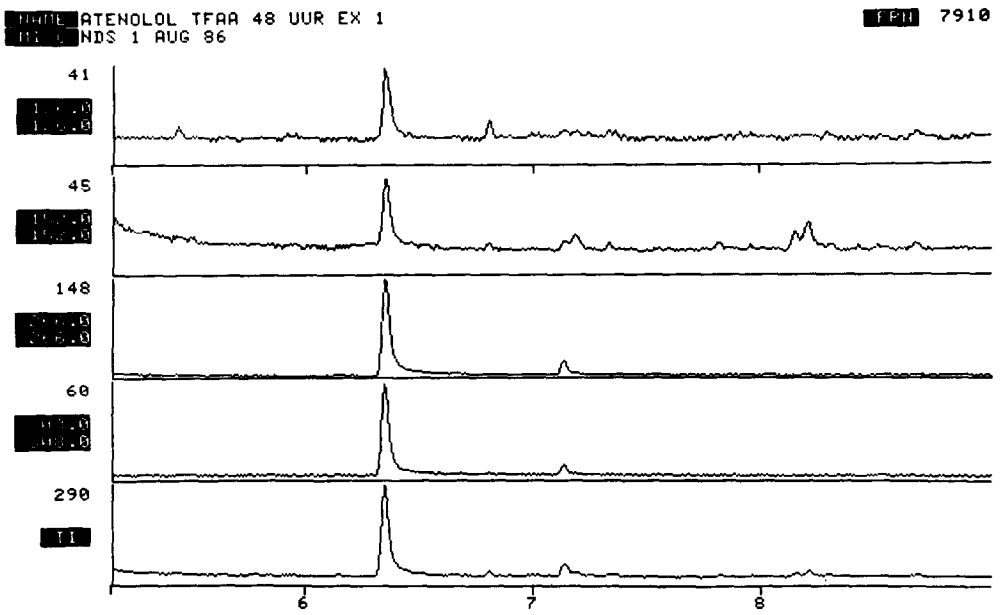

Figure 5
Mass fragmentogram and TLC of a urine extract 48 h after atenolol administration (Extrelut-1 extraction); peak with retention time 6.38 min is atenolol-di-TFA.

Table 3
Detection limit (ng ml^{-1}) of several β-blockers analysed by CGC–NPD and GC–MS

		Liquid	Extrelut-1	Sep-Pak
Propranolol	a.	200	500	330
	b.	6	10	4
Atenolol	a.	200	500	330
	b.	4	10	15
Oxprenolol	a.	100	500	330
	b.	10	20	15
Pindolol	a.	400	1000	700
	b.	10	20	15
Metoprolol	a.	200	500	330
	b.	10	20	6
Alprenolol	a.	100	500	330
	b.	6	10	4

a. CGC–NPD.
b. GC–MS (m/z = 308 and 266).

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