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Detection of β-blockers in urine by solid-phase extraction–supercritical fluid extraction and gas chromatography–mass spectrometry

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Abstract

The most convenient way to perform supercritical fluid extraction (SFE) of liquid sample matrices is to combine it with solid-phase extraction (SPE). β-Blockers from urine were collected on an Empore disc, which was then placed into an extraction cell for derivatization and SFE. SPE recovery was best at pH 10. Effects of temperature, pressure and volume of pyridine on the acetylation and SFE processes were studied. Without acetylation the β-blockers were not significantly soluble in CO_2. SFE temperatures of 70°C and 150°C together with 200 μl of acetic anhydride and 400 μl pyridine gave the best results. With the SPE–SFE–GC–MS method developed here, β-blockers like oxprenolol, metoprolol and propranolol could easily be detected in urine samples, and the limit of detection (LOD) for these compounds was found to be 20 ng/ml, 30 ng/ml and 40 ng/ml, respectively.

Keywords: Supercritical fluid extraction; Solid-phase extraction; β-Blockers; Oxprenolol; Metoprolol; Propranolol

1. Introduction

β-Blockers are used in the treatment of various cardiovascular disorders including angina pectoris, cardiac arrhythmia and hypertension. They are toxic, however, and most have only a narrow therapeutic range. Unfortunately, they have also been misused as doping agents in sports, where they have been forbidden since 1988 by the Medical Commission of the International Olympic Committee. Typically, oral doses of 5–100 mg of these drugs have been administered to decrease the heart rate and muscular tremor during archery, billiards and riflery competitions [1,2].

Determination of β-blockers, especially in biological fluids (urine and serum), is difficult owing to their low concentrations and those of their metabolites relative to the high concentrations of endogenous compounds in the matrix. β-Blockers have been determined by liquid chromatography (HPLC) with UV or fluorescence detection [3], electrophoretic methods [4] and gas chromatography–mass spectrometry (GC–MS) [5]. MS detection coupled with GC gives good sensitivity and selectivity, with a great deal of structural information for identification purposes to make the analysis more reliable.

Several extraction (and clean-up) methods for β-blockers from urine have been applied [6,7]. While

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liquid–liquid extraction (LLE) fails to produce good recoveries for compounds varying widely in polarity, solid-phase extraction (SPE) is superior in giving good recoveries for hydrophilic and hydrophobic analytes at the same time [7,8]. Pretreatment of the sample by SPE and LLE usually includes a separate deproteinization step, and derivatization if GC is used. Sometimes with LLE, back-extractions of the drugs into the aqueous phase are used to clean up the sample.

Thanks to the special properties of supercritical fluids, supercritical fluid extraction (SFE) is much faster and in many cases more efficient than conventional solvent-extraction methods. SFE can be run either in static or in dynamic mode. In static mode the sample in the extraction cell is in contact with fluid which is pressurized at a certain temperature without any flow through the system. After static extraction analytes are swept out dynamically. In dynamic mode the sample is extracted at a certain temperature and pressure with constant fluid flow-rate. Extracted analytes can be collected at the restrictor exit from the fluid expanding to atmospheric pressure by using a suitable adsorbent trap, collection into a solvent or a cold trap. SFE is an excellent method for extracting organics from solid sample matrices such as soil [9], sediment [10], flyash [11], various sorbent materials [12] and food products [13]. Already, SFE has become an alternative method to Soxhlet extraction [14,15] and extraction with sonication [15]. Just recently, SFE equipments with autosamplers have become commercially available; with better repeatability, SFE is now more reliable and should quickly find its way into routine use.

Despite the excellent record of SFE in extracting organics from solid supports, little attention has been paid to extractions from liquid sample matrices. This is probably because the sample matrix can easily be flushed out with the analytes if the flow-rate (pressure) is too high or the sample volume too large. With aqueous samples, moreover, the temperature cannot be raised excessively, in order to ensure that only two phases (liquid and supercritical fluid) exist. It is more difficult to extract polar and hydrophilic compounds from water than from solid matrix with CO_2, and since the use of modifiers and fluids more soluble than CO_2 in water is ruled out, the capability of SFE to work with liquid matrices is limited.

The combination of SPE and SFE would seem to be a promising solution for the extraction of compounds from aqueous matrices [16–18]. The gaseous properties of CO_2 make it easy to connect SFE to a gas chromatograph, and an SPE–SFE–GC on-line system offers a powerful tool for complex samples.

The purpose of our work was to evaluate the capability of SFE to extract β-blockers from Empore C_{18} solid-phase extraction discs, which had been used to collect those drugs from urine samples. To minimize the number of pretreatment steps, β-blockers were derivatized right in the SFE extraction vessel. Acetic anhydride was used as the derivatization reagent. After SPE–SFE the acetylated drugs were analysed by GC–MS. The best conditions found for the extraction and derivatization, and the results for clinical urine samples obtained with the developed SPE–SFE–GC–MS method, are presented.

2. Experimental

2.1. Instrumentation

Empore C_{18} extraction discs (Varian, Harbor City, CA, USA) of 47 mm diameter were used in all experiments. Supercritical fluid extractions were performed with a Suprex Prep Master using a Suprex 5-ml extraction vessel. A 10-cm linear fused-silica capillary with 30 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) was used as a restrictor and was connected to the outlet capillary with a 1/16-inch Swagelok male to male connector. Extracted analytes were collected into 3.5 ml of methanol placed in a 7.5-ml glass screw-top vial. The collection vial was kept in an aluminium block to prevent solvent cooling.

Gas chromatographic analyses were carried out with a Hewlett-Packard Model 5890 gas chromatograph. The column was a 14-m HP-5 with 0.2 mm I.D. and 0.33 μm film thickness. A deactivated DPTMDS retention gap (HNU-Nordion, Helsinki, Finland) 1.5 m in length and of 0.32 mm I.D. was

connected to the column with a glass pressfit connector (Beat Schilling, Zürich, Switzerland). All injections were made at 30°C with an HP on-column injector. Constant pressure (60 kPa) mode was selected for GC analysis, and the oven was programmed from 30°C (2 min) to 220°C at 15°C/min, from 220°C (1 min) to 260°C at 5°C/min and from 260°C to 320°C (3 min) at 15°C/min. The gas chromatograph was connected to an HP Model 5989A mass spectrometer, and the GC–MS system was controlled through an HP ChemStation program installed in the computer.

2.2. Materials and reagents

The β-blockers were alprenolol hydrochloride, oxprenolol hydrochloride, (±)-metoprolol (±)-tartrate salt (all from Sigma, St. Louis, MO, USA) and (±)-propranolol hydrochloride (Aldrich, Milwaukee, WI, USA). Analytical-grade acetic anhydride (Merck, Darmstadt, Germany) and pyridine (Merck) were distilled just before use. Methanol and methylene chloride were HPLC grade (Lab-Scan, Dublin, Ireland). Buffers used for the SPE recovery study were borax/HCl at pH=8 (Merck), 0.01 *M* borax solution ($Na_2B_4O_7 \cdot 10H_2O$; p.a., Merck) at pH=9 and boric acid/KCl–NaOH at pH=10 (Merck). For urine samples the pH of the 0.01 *M* borax buffer was adjusted to 10 with 0.1 *M* NaOH (Merck) and this was used instead of the buffer (pH=10) employed in the SPE trapping study. Distilled water was ion-exchanged with a Water-I system (Gelman Sciences, Ann Arbor, MI, USA) before use. Helium was used in GC as a carrier gas, and SFE-grade carbon dioxide (Air Products and Chemicals, Allentown, PA, USA) was used for all SFE extractions.

The tablets, taken by healthy young volunteers, contained 25 mg of propranolol (Propral, Medipolar), 40 mg of oxprenolol (Trasicor, Ciba) and 50 mg of metoprolol (Seloken, Astra). The tablets were given one at a time, and another drug was not taken before the urine of the volunteer had returned to the drug-free state. The β-blockers were administered after overnight fasting. The urine samples were collected 0–4 h after intake to minimize the amount of metabolites.

2.3. Preparation of solutions

Two stock solutions were prepared: one (A) containing 5 mg/ml each of oxprenolol, metoprolol and propranolol in methanol and the other (B) containing 5 mg/ml of alprenolol in methanol.

For the SPE and SFE recovery studies, 150 μl of solution A were diluted with methanol to a final volume of 5 ml (C). A 50-μl sample of solution C was used for each analysis. For preparation of the internal standard, 150 μl of B was added to the test tube and evaporated to dryness, after which 200 μl of acetic anhydride and 120 μl of pyridine were added, and the mixture was heated for 1 h at 80°C. This was again evaporated to dryness, and 5 ml of methanol were added to obtain solution D. The amount of solution D added as internal standard was 50 μl. For measurement of 100% recovery, 50 μl of C (and 50 μl D) were acetylated in a test tube and dissolved in 200 μl of methylene chloride–methanol (9:1, v:v). The amount of analyte detected by GC–MS was regarded as the 100% recovery.

For preparation of linear calibration plots, 50, 100, 150 and 200 μl of solution A were taken, 150 μl of solution B were added to each, and the mixture was diluted with methanol to 5 ml. Blank urine samples (2 ml) were spiked with 50 μl of these solutions and analysed by SPE–SFE–GC–MS. In the analysis of urine samples (2 ml), 3 ml of buffer solution were added to adjust the pH, and 50 μl of ISTD solution (E), which was obtained by diluting 150 μl of B with methanol to a volume of 5 ml, were added to the urine before determination.

2.4. Extractions

Immediately before use, while in the vacuum filtering device, the Empore disc was washed successively with 20 ml of methanol, 20 ml of water and 10 ml of the buffer solution used to adjust the pH of the sample. Care was taken not to let the disc dry between these steps. After the sample (5 ml) was introduced to the disc, 2×5 ml of buffer solution were used to wash the sample tube and filtering system. All of the 15-ml samples (2×5 ml washing buffer included) were slowly filtered in 15 min. Finally, the Empore disc was dried in full vacuum

for 7 min and transferred to the SFE extraction vessel.

Acetic anhydride and pyridine were added to the extraction vessel. After the pressure and temperature were stabilized, SFE was run in static mode for 15 min to allow time for the acetylation reaction to occur. Searching for the right conditions, 40 g of CO_2 were used for each dynamic extraction.

After completion of SFE, the collection solvent and extracted reagents were evaporated to dryness under nitrogen flow at 80°C. The vial was allowed to cool, 200 μl of methylene chloride–methanol (9:1, v/v) were added, and the sample was analysed by GC–MS (1 μl on-column). In recovery studies, 50 μl of D (I.S.) were added after SFE, before evaporation to dryness.

3. Results and discussion

GC–MS runs for recovery studies were done in scan mode from 50 to 500 u. Mass spectra of acetylated alprenolol (I.S.), oxprenolol, metoprolol and propranolol were quite similar, with the most intensive ions of mass-to-charge ratios 56, 72, 98 and 200. Ions 72, 158 and 200 were selected for SIM analysis of urine samples. In the case of recovery studies (scan) and urine samples (SIM), the chromatogram for ion 200 was extracted from TIC and used for quantitation (all calculations based on ion 200 chromatograms).

Although the retention times of the acetylated β-blockers were obtained using standards, the analytes from clinical samples were identified not only by the retention time but also by comparing the relative intensities of the three ions (72, 158 and 200) with the relative intensities of those ions in the mass spectra recorded.

To test the capability of the Empore disc to retain β-blockers at different pHs, 50 μl of solution C were spiked into 5 ml each of the three buffer solutions with pH 8, 9 and 10 (see Section 2). β-Blockers of these three samples were extracted from SPE discs with SFE using 500 atm pressure at 70°C, as in the preliminary experiments [19]. Because of the derivatization taking place at the same time with the extraction, a 15-min static period was allowed at the beginning of the extraction. Longer static periods were not tested, although slightly better recoveries might have been achieved by increasing the derivatization time. However, 15 min was expected to be enough to produce good recoveries in a reasonable time.

A pH above 10 was not used because, according to the manufacturer, the upper pH limit for Empore discs is 7. Higher pHs are recommended only for short time periods. The Empore discs nevertheless seemed to withstand higher pHs very well. Because the pK_a values of the analytes vary from 9.2 to 9.6, better recoveries would probably not have been achieved above pH 10, which was the best pH studied (Fig. 1). For the SFE efficiency studies, the effect of the temperature on the recovery was tested. The sample was spiked on a small piece of filter paper (6×6 cm) wich was used to simulate the Empore disc. After the evaporation of the solvent, the paper was inserted into the extraction vessel. Temperatures of 70°C and 150°C gave the best results.

To increase the SFE recovery, basic pyridine was added to act as a solvent and catalyst in the acetylation process. Pyridine improved the derivatization process (or extraction) more in SFE than in a normal test tube, as can be seen from Fig. 2. After these experiments it was necessary to find out if the low recoveries were due to SFE or the acetylation process. β-Blockers were acetylated in a test tube and spiked onto the filter paper, which was inserted into the extraction vessel. The recoveries of the

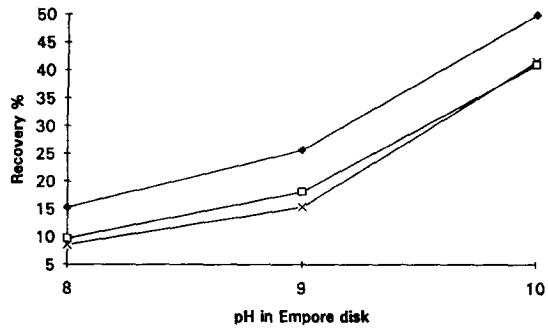

Fig. 1. Effect of sample solution pH on the capability of the Empore disc to retain β-blockers. Drugs were spiked into the buffer solution and eluted from the disc with SFE using CO_2 at 500 atm and 70°C. Derivatization with 200 μl acetic anhydride was carried out simultaneously in the extraction vessel. ×= Oxprenolol, □=metoprolol and ◆=propranolol.

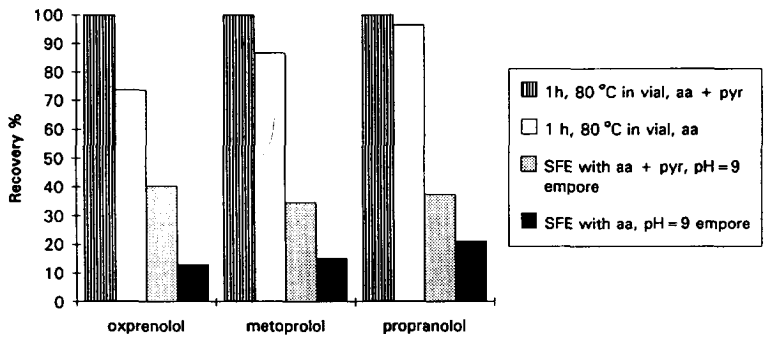

Fig. 2. Recoveries of β-blockers acetylated in a test tube and with SFE. Either 200 μl acetic anhydride or 200 μl acetic anhydride plus 120 μl pyridine were used. SFE at 500 atm and 70°C.

acetylated β-blockers are seen as a function of SFE temperature in Fig. 3. At 150°C the recovery was almost 90% for all of the compounds. This shows that, after acetylation, β-blockers can be efficiently extracted and collected.

The extractability of underivatized β-blockers with CO_2 was also studied at different temperatures (Fig. 3). From Fig. 3 it can be seen that at low extraction temperatures β-blockers are not soluble in CO_2, and with acetylation the solubility is greatly enhanced. At higher temperatures the recoveries of underivatized compounds were increased, probably due to the higher vapour pressures (thermodesorption). Additionally, it is now clear that pyridine must be added to increase the solubility of β-blockers in CO_2 enough so that they can be efficiently acetylated and/or to increase the speed of the acetylation process.

Acetylation in SFE gave better results at higher temperatures, probably due to the faster diffusion of the analytes. Comparing the recoveries in Fig. 3 with the low recoveries in Fig. 2 revealed that the derivatization in SFE was incomplete. The efforts to improve the acetylation by increasing the acetic anhydride volume failed because of serious restrictor blocking problems. Silica restrictors also became fragile and broke during the extraction.

An attempt was made to improve the derivatization by increasing the pyridine volume, and the best recovery was obtained with 400 μl pyridine (Fig. 4). In SFE, pyridine can also act as a modifier to release analytes from the matrix, after which they are free to be acetylated. The lower recovery in Fig. 4 than in

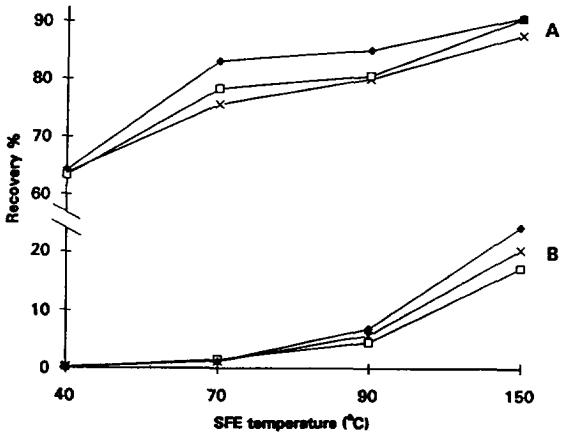

Fig. 3. Recoveries of acetylated (in a test tube) β-blockers (A) and underivatized β-blockers (B) extracted with CO_2 at 500 atm and at various temperatures. β-Blockers were spiked onto filter paper. ×=Oxprenolol, □=metoprolol and ♦=propranolol.

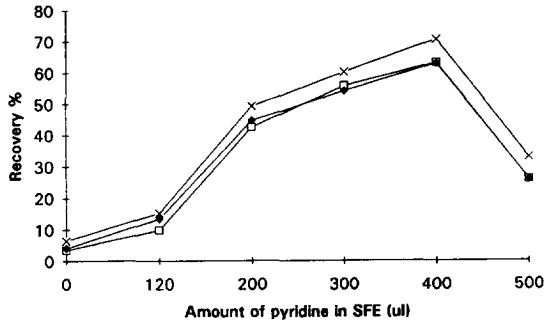

Fig. 4. Recovery of β-blockers derivatized in SFE with 200 μl of acetic anhydride in the presence of different volumes of pyridine. β-Blockers were spiked onto an Empore disc. SFE was performed at 500 atm, at 70°C in the static and 150°C in the dynamic period. ×=Oxprenolol, □=metoprolol and ♦=propranolol.

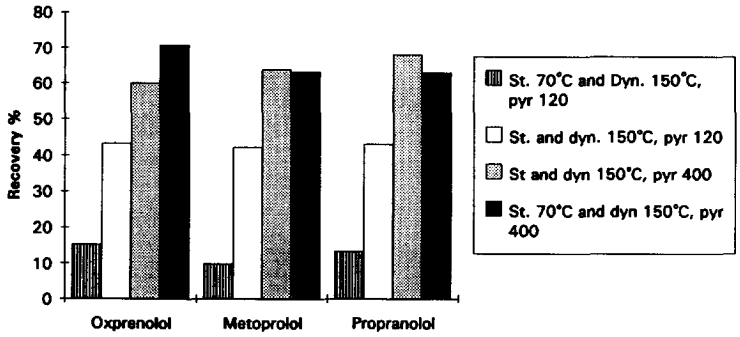

Fig. 5. Recovery of spiked (onto an Empore disc) β-blockers using two amounts of pyridine and a temperature of 70°C or 150°C during the static period. SFE at 500 atm, dynamic step at 150°C, derivatization with 200 μl acetic anhydride.

Fig. 3 indicates that the acetylation was still not complete or that the Empore disc more effectively retains analytes than does filter paper during SFE. A dramatic drop in recovery with higher pyridine volumes than 400 μl was probably due to the changes in SFE conditions. The excess of pyridine might also prevent the β-blockers from being acetylated. Lower CO_2 pressures during the acetylation did not produce higher recoveries.

Finally, with 200 μl of acetic anhydride and 400 μl or 120 μl of pyridine with dynamic mode at 150°C, the two best temperatures, 70°C and 150°C, were checked for the static period. Fig. 5 shows the difference between these two temperatures to be greatest for 120 μl pyridine, indicating that with small volumes of acetylating reagent, the derivatization process is slow. The probable reason for this is that some of the reagents are pushed into the capillary after the extraction vessel when this is pressurized. Moreover, 120 μl of pyridine is not enough to enhance the solubility of β-blockers so that they can efficiently react with acetic anhydride. At higher temperatures, diffusion rates are faster, and also β-blockers are more efficiently released from the matrix (see Fig. 3), leading to faster acetylation. This explains the higher recoveries at temperatures of 150°C. With 400 μl of pyridine the difference in the recoveries between 70°C and 150°C is small, and both temperatures can be used. For urine samples, 150°C was selected as the temperature of the static period. The reason why in all experiments the best recoveries were obtained for propranolol is not clear. In the literature, too, the best recoveries in solid-phase extraction with C_2-phase are reported for propranolol [8].

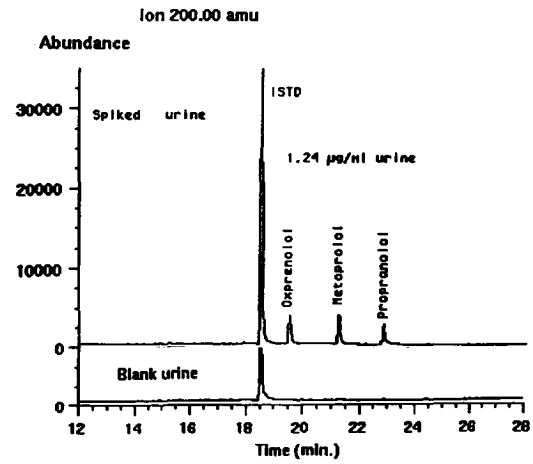

Fig. 6. GC–MS ion chromatogram of blank urine (lower trace) and spiked urine (upper trace) both extracted using an Empore disc at pH 10 and SFE. The sample was acetylated in the extraction vessel. SFE at 500 atm and 150°C. Acetic anhydride (200 μl) and pyridine (400 μl) were used for derivatization.

For urine samples, 0.01 *M* borax/NaOH buffer (pH=10) was used because a better buffer capacity was achieved than with the buffer used in the SPE recovery study. The GC–MS ion chromatogram for blank urine showed that no disturbing peaks were eluted with the same retention time as the analytes (Fig. 6). After extraction of the blank urine sample, different amounts of β-blockers were spiked into the blank urine (1–5 μg/ml urine), and calibration curves were generated after extracting and analysing

those samples. Correlation factors for all the β-blockers were 0.999 or better with four-point calibration. Fig. 6 shows the chromatogram of a spiked urine sample and Fig. 7, the chromatograms of clinical samples containing oxprenolol or metoprolol. For oxprenolol (0.22 μg/ml urine) the signal-to-noise ratio (*S/N*) was 35.0, while for metoprolol (1.38 μg/ml urine) it was 73.2. The limit of detection (LOD), corresponding to a signal-to-noise ratio of 2, for oxprenolol, metoprolol and propranolol was 20 ng/ml, 30 ng/ml and 40 ng/ml, respectively. The concentration of propranolol was so low that it could not be detected in the 2-ml urine sample. For the propranolol sample, a volume as high as 5 ml of urine should have been used.

Although SPE–SFE seems to be a very efficient method of sample preparation, more experiments are needed to determine the overall efficiency and to optimize the length of the static and dynamic periods in SFE.

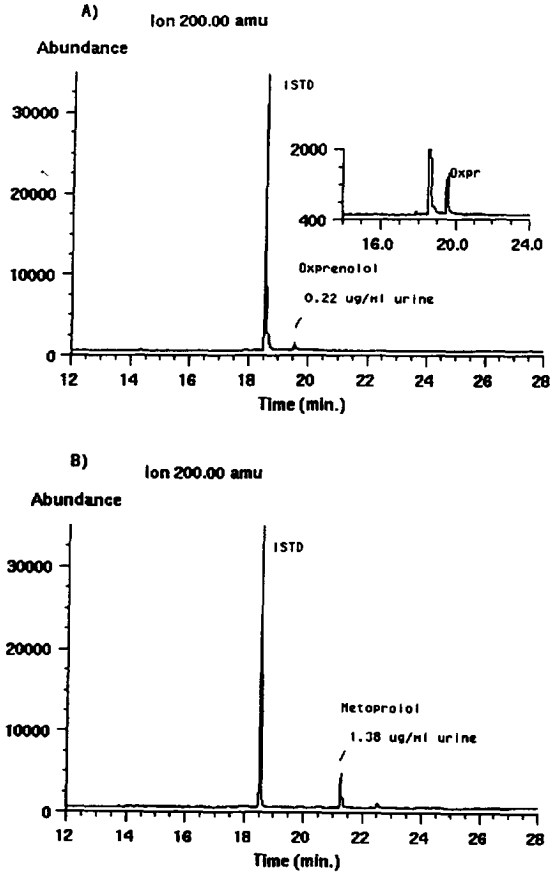

Fig. 7. GC–MS ion chromatogram of β-blockers collected from clinical urine samples 0–4 h after intake. (A) Oxprenolol and (B) metoprolol. SFE conditions and acetylation as in Fig. 6.

4. Conclusions

The SPE–SFE–GC–MS method for the determination of β-blockers from urine samples proved to be very convenient, and steps such as enzymatic hydrolysis (deproteinization), centrifugation and some evaporation steps, which are normally involved in the preparation of urine samples, could be eliminated. Also, the total time needed for the analysis could be reduced through simultaneous derivatization and SFE.

β-Blockers oxprenolol, metoprolol and propranolol were easily detected in urine samples at the ng/ml level. More polar solid-phase extraction disc materials might produce an improvement of this method.

Acknowledgments

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