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Screening and determination of β-blockers, narcotic analgesics and stimulants in urine by high-performance liquid chromatography with column switching

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Abstract

A fast method is described for the screening of eleven β-blockers, two narcotic analgesics and two stimulants in urine by HPLC with column switching. The urine sample (100 μl), buffered to pH 9–9.5, is injected onto a short extraction column packed with CN stationary phase. The extraction column is flushed with water for 2.5 min to elute polar matrix components to waste. The retained components are then backflushed by means of a six-port valve onto the ODS analytical column where they are separated. Phosphate buffer pH 3.0 and acetonitrile were used as mobile phase. Gradient elution was applied in the screening method to improve separation. Detection was performed with a diode-array detector at 220, 235 and 300 nm. Recoveries were near 100%, precision was excellent and sensitivity about 0.25 μg/l. To speed up the quantitative analysis, the same method but with isocratic elution was successfully applied to the determination of acebutolol and metoprolol in urine samples collected 4 h after administration of the compounds as single doses.

1. Introduction

Screening and determination of β-blockers, narcotic analgesics and stimulants in biological samples are required in many areas, including doping control, forensic analysis and toxicology. In doping control, the most common method for screening of these drugs in urine is liquid–liquid extraction (LLE) or solid-phase extraction (SPE) for sample preparation, followed by derivatization of the polar functional groups and analysis of the extract by gas chromatography–mass spectrometry (GC–MS). Advantages of the GC–MS method are its sensitivity and the easy identification of compounds and their metabolites from their mass spectra. However, the poor GC properties and instability of some derivatives may limit the use of GC–MS [1–4]. Other techniques used for screening of these compounds are high-performance liquid chromatography (HPLC) [5,6], thin-layer chromatography [7], capillary electrophoresis [8] and micellar electrokinetic chromatography [9,10].

The need for more efficient, faster and automated systems for drug analysis has encouraged a search for methods in liquid chromatography

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allowing direct injection of the sample onto the column without prior sample preparation. Column switching [11], micellar chromatography [12], special stationary phases [13] and combinations of these methods [14] have been used to analyse various drugs, including β-blockers, narcotic analgesics and stimulants, in biological fluids. Recently, a column-switching method was applied to the screening of diuretics in urine [15,16].

In this paper we report column-switching methods for the screening and determination of β-blockers, narcotic analgesics and stimulants in urine. A short dry-packed column containing CN stationary phase was used for the extraction of the drugs from urine, while a silicone polymer-coated silica gel modified with octadecyl groups provided the stationary phase in the analytical column. Gradient elution was used to improve the resolution in screening and isocratic conditions were used to speed up determinations.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of a Hewlett-Packard Model 1090 instrument equipped with a 1040 diode-array detector (DAD), a computer, a disc drive unit, an integrator, a printer (Hewlett-Packard, Avondale, PA, USA), an LKB 2150 HPLC pump (Bromma, Sweden) and a Valco 6-port valve. The volume of the sample loop was 100 μl. The complete column-switching system is shown in Fig. 1. The metabolites of acebutolol and metoprolol were identified by GC–MS using a Hewlett-Packard Model 5890A gas chromatograph and a Hewlett-Packard Model 5989A single-stage quadrupole mass spectrometer. The experimental conditions have been described earlier [6].

2.2. Chemicals

Acebutolol hydrochloride, atenolol, alprenolol hydrochloride, bopindolol, labetalol hydrochloride, metoprolol tartrate, nadolol, oxprenolol hydrochloride, pindolol, propranolol hydrochloride and timolol maleate and β-glucuronidase/arylsulfatase mixture from *Helix pomatia* were obtained from Sigma (St. Louis, MO, USA). Amphetamine, codeine, ephedrine and morphine were obtained from The National Bureau of Investigation, Crime Laboratory in Helsinki. HPLC-grade acetonitrile and boric acid were from Merck (Darmstadt, Germany), and HPLC-grade concentrated phosphoric acid was from Fisher (Fair Lawn, NJ, USA). All chemicals were of analytical grade.

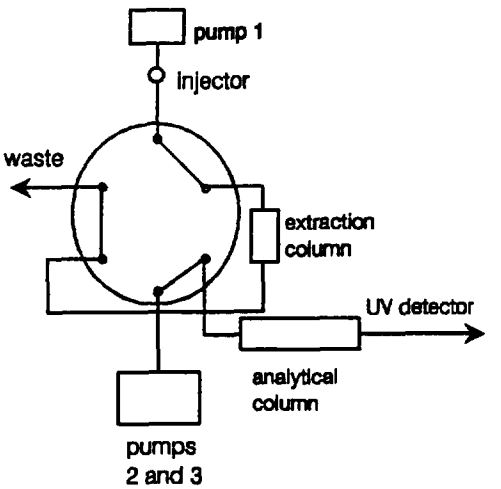

Fig. 1. Schematic representation of the column-switching system. The valve is in position 1.

2.3. Standard solutions and urine samples

Standard stock solutions of drugs were prepared by dissolving appropriate amounts of the drugs in methanol to give a concentration of 5 mg/ml. The solutions were protected from light and stored at 4°C. Drug-free urine was obtained from healthy volunteers. The urine samples were obtained from healthy volunteers 4 h after single oral doses of acebutolol and metoprolol. Working standard solutions in water and spiked urine samples were prepared by adding appropriate amounts of stock solution. Urine samples were hydrolysed by adding 10 mg of β-glucuronidase/arylsulfatase mixture to 1.0 ml of urine and incubating at 37°C overnight [1,5]. Urine sam-

ples were then buffered to pH 9–9.5 by mixing them 1:1 with 0.2 *M* boric acid buffer solution in which the pH was adjusted to 9.5 with 5 *M* sodium hydroxide. Before injection urine samples were centrifuged at 2000 *g* for 5 min and filtered.

2.4. Chromatographic conditions

The ODS column used for the analytical separation was Capcell Pak C_{18} UG-120 (Shiseido, Japan, 250 × 4.6 mm I.D., 5 μm). The extraction column (10 × 4.6 mm I.D.) was dry-packed with cyanopropyl-modified silica gel (Spherisorb, 5 μm).

The eluent for the extraction of polar matrix compounds (pump 1) was HPLC-grade water. The eluent for the backflushing and analytical separation (pumps 2 and 3) consisted of a gradient or a mixture of 0.05 *M* phosphate buffer (pH 3.0) and acetonitrile. The buffer was prepared by diluting 3.4 ml of concentrated phosphoric acid in 1000 ml of water. The pH was adjusted with 5 *M* NaOH. Both eluents were filtered and degassed before use. The gradient used for the backflushing and analytical separation in the screening procedure was: initial acetonitrile 3%, increased to 30% at 10 min and 40% at 18 min. In determinations the concentration of acetonitrile in the mobile phase was 22 and 23% for acebutolol and metoprolol, respectively. The flow-rate of the extraction eluent was 1.25 ml/min and that of the analytical eluent 1 ml/min. The column temperature was ambient. The diode-array detector was set to monitor the signals at 220, 235 and 300 nm.

2.5. Column-switching procedure for screening

Step 1 (0–2.5 min). The valve is in position 1 (Fig. 1). The urine sample is injected onto the extraction column with water as eluent from pump 1. The drugs of interest are retained by the extraction column while polar matrix components are eluted to waste.

Step 2 (2.5–17.5 min). The valve is switched to position 2. A gradient of the mobile phase from pumps 2 and 3 is passed through the extraction column in the backflush mode and elutes the retained components onto the analytical column, where they are separated.

Step 3 (17.5–30 min). The valve is switched to position 1. The extraction column and the analytical column are allowed to stabilize for 12 min before the next injection.

2.6. Column-switching procedure for determinations

Step 1 (0–2.5 min). The valve is in position 1. The urine sample is injected onto the extraction column as in the screening procedure.

Step 2 (2.5–3.75 min). The valve is switched to position 2. An isocratic mobile phase is passed through the extraction column in the backflush mode and the retained components are eluted onto the analytical column.

Step 3 (3.75–5 min). The valve is switched to position 1 and the extraction column is allowed to stabilize for 1.5 min

Step 4 (0–2.5 min). The next sample is injected onto the extraction column.

2.7. Recovery

Recoveries were calculated by comparing the peak heights of spiked samples passed through both the extraction and analytical columns with the peak heights of standards of the same concentration injected directly onto the analytical column.

3. Results and discussion

3.1. Screening procedure

Sample preparation and extraction

A cyanopropyl modified silica gel was used as stationary phase in the extraction column. The retention of standard solutions and urine matrix components on the column was investigated using water as eluent. No elution of drugs was observed when the extraction column was flushed for 10 min, indicating that the drugs were well retained. In the backflush mode, with the

analytical mobile phase used for elution, all compounds were eluted in about 1 min. When 50 μl of drug-free urine was injected onto the extraction column, the minimum flushing time required to remove the most polar urine matrix components was ca 2.5 min. However, when the analytical column was connected to the system and the gradient used for the backflushing and separation, interfering peaks appeared in the chromatogram. It was known from our previous study [15] that endogenous urine matrix components are less strongly retained on the ODS column when the pH of the sample is increased. The urine sample was accordingly buffered to pH 9–9.5 with boric acid buffer, which resulted in a considerable decrease of urine matrix components in the chromatogram. When standard solutions were buffered to pH 9.5, analyte was lost probably due to adsorption on the test tubes. No losses were observed with spiked urine samples, however. The recoveries from a spiked and buffered urine sample approached 100% for most of the compounds. Fig. 2 shows a chromatogram of a urine blank with the detector set at 220 nm. The peaks at the end of the chromatogram are hydrolysed endogenous compounds in the urine.

Analytical separation and detection

The ODS column used for the analytical separation was Capcell Pak C_{18} UG-120. In Capcell Pak columns the silica gel is coated with a silicone polymer before the functional groups are bonded to the surface in order to suppress the silanol effects when basic analytes are chromatographed [17–19]. In this study, a gradient of acidic phosphate buffer pH 3.0 and acetonitrile was used for the analytical separation. The concentration of acetonitrile at the beginning of the gradient was adjusted to 3% to enrich the early eluting morphine and atenolol at the front of the analytical column. Fig. 3 shows a chromatogram of a standard solution containing 11 β-blockers, 2 narcotic analgesics and 2 stimulants with the detector set at 220 nm. All compounds except acebutolol and timolol could be separated from each other under the chromatographic conditions described.

Three wavelengths, 220, 235 and 300 nm, were used to monitor the eluting compounds. Most of the drugs have a strong absorbance at 220 nm. Acebutolol was detected at 235 nm and timolol at 300 nm. Amphetamine and ephedrine have their absorption maximum at 210 nm, but this wavelength was too low due to the background noise.

Figs. 4 and 5 show chromatograms of urine samples obtained 4 h after oral administration of acebutolol and metoprolol, respectively, to healthy volunteers. The peaks eluting before the parent compound were identified as metabolites of acebutolol and metoprolol. The samples were

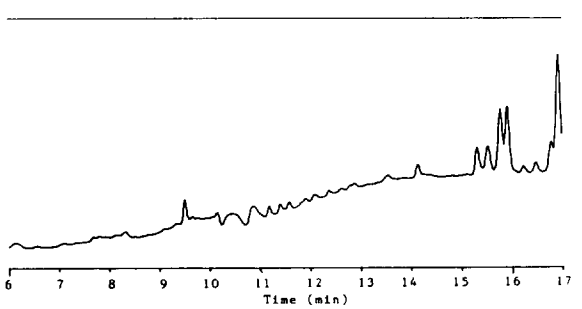

Fig. 2. Chromatogram of a blank urine sample. Detection is at 220 nm and 0.05 AUFS.

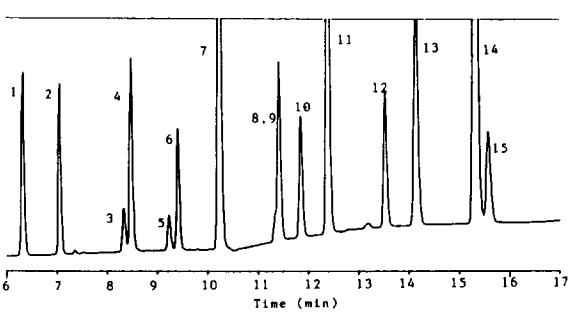

Fig. 3. Chromatogram of a standard solution (5 μg/ml) containing β-blockers, narcotic analgesics and stimulants. Detection is at 220 nm and 0.12 AUFS. Peaks: 1 = morphine; 2 = atenolol; 3 = ephedrine; 4 = codeine; 5 = amphetamine; 6 = nadolol; 7 = pindolol; 8 = timolol; 9 = acebutolol; 10 = metoprolol; 11 = bopindolol; 12 = oxprenolol; 13 = labetalol; 14 = propranolol; 15 = alprenolol.

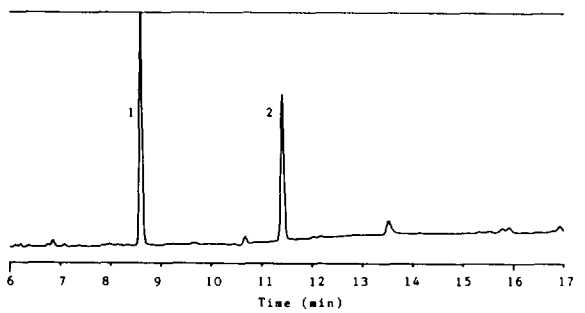

Fig. 4. Chromatogram of a urine sample 4 h after administration of a 100-mg dose of acebutolol. Peaks: 1 = metabolite of acebutolol; 2 = acebutolol (6.9 μg/ml). Detection is at 235 nm and 0.22 AUFS.

confirmed to contain the metabolites of acebutolol and metoprolol by GC–MS as described earlier [6].

3.2. Quantitative analysis of urine samples

To accelerate the quantitation of acebutolol and metoprolol in urine samples the column-switching procedure used for the screening was modified by using isocratic instead of gradient conditions for the separation. The extraction step was the same as in the screening procedure, but 1.25 min after the valve was switched to the backflush position it was again switched to the extraction position to re-equilibrate the extraction column with water. After 1.25 min re-equilibration the next sample was injected onto the extraction column. With this procedure, a new sample or standard solution could be injected to the system every 5 min. The concentration of acetonitrile in the mobile phase was adjusted so that the retention time of the compound in the analytical column was between 5.5 and 6 min. The amounts of acebutolol and metoprolol found 4 h after administration of a single dose are reported in Figs. 4 and 5.

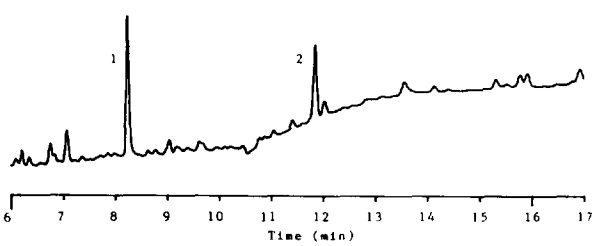

Fig. 5. Chromatogram of a urine sample 4 h after administration of a 50-mg dose of metoprolol. Peaks: 1 = metabolite of metoprolol; 2 = metoprolol (0.7 μg/ml). Detection is at 220 nm and 0.05 AUFS.

3.3. Validation

The precision and accuracy of the screening method were evaluated by performing six replicate analyses of a spiked urine sample containing 5 μg/ml of each of the drugs investigated. For most of the compounds the recoveries were excellent and relative standard deviations ranged from 1 to 3%. The coefficients of variation of absolute retention times were under 0.2%. Determination limits (signal-to-noise ratio 3) when a 100-μl urine sample (diluted 1:1) was injected are about 0.25 μg/ml.

The quantitation of acebutolol and metoprolol in urine was done using the external standard method and peak heights. The calibration curves were linear at least from 0.4 to 15 μg/ml. The coefficients of variation of four replicate analyses were under 2%.

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