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Determination of Metoprolol and Its Metabolites in Human Urine by Capillary Isotachophoresis

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Abstract

The separation and determination of metoprolol and its metabolites M I (metoprolol acid), M II (2-hydroxy-3-(4-metoxyethylphenoxy)-propanoic acid, M III (a-hydroxymetoprolol) and H 105 (O-desmethylmetoprolol) in human urine by capillary isotachophoresis were investigated. Metoprolol and metabolites M I, M III and H 105 were separated by cationic isotachophoresis in the electrolyte system sodium acetate buffer (pH 5.0) (c_L=10 mM) -β-alanine. Metabolite M II was separated using the anionic electrolyte system histidine hydrochloride buffer (pH 6.0) (c_L = 10 mM) -morpholinoethane-sulfonic acid. Endogenous and the possible exogenous compounds were almost totally removed from urine by solid-phase extraction using a SepPak C_{18} cartridge. The recovery of compounds varied from 84.6 to 95.8%. The linear calibration range was studied for eventual application of the method to real urine samples. Limit of determination was 0.5 mg/ml.

Keywords: human urine, capillary isotachophoresis

Introduction

Pharmacokinetic studies in men have demonstrated that metoprolol is eliminated from the human body mainly in the form of metabolites. Only a minor fraction of the given dose is recovered as an unchanged drug in human urine. Four metabolites were identified in urine, two of them H 105 and M III passes β-blocking activity. Metabolite M III accounts for 10% of the dose in urine. Metabolite H 105 is rapidly oxidized to an inactive aminoacid (M I) and recovered in only minor amounts in human body fluids. The other two metabolites M I and M II have no pharmacological activity, accounting for about 65 and 10% of the oral dose, resp. [1]. There are GC-MS [2-4] and HPLC [5-10] method for the assay of metoprolol and its metabolites in the literature.

We present a simple ITP method for the major metabolite metoprolol acid (M I), involving direct injection of urine. The method allows the assay of metoprolol and α-hydroxymetoprolol (M III) which is a relatively minor metabolite of metoprolol. By using a solid-phase extraction, the metabolite M II could be included and the same four compounds were measured in urine.

Experimental

Apparatus

Isotachopherograms were obtained by the use of a Villa Labeco ZKI 02 isotachophoretic analyzer equipped with a conductivity detector. The analytical column (90 mm x 0.3 mm I.D.) was connected with a preseparation column (90 mm x 0.8 mm I.D.). Measurement data were acquired with a PC/AT with a 12-bit A/D-D/A converter at 100 Hz sampling frequency. For solution pH measurement, a model OP-211 pH meter (Radelkis, Hungary) with a combined glass electrode was employed.

Materials

All chemicals were of the highest quality commercially available. Deionized, redistilled water was

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used in the preparation of the electrolyte systems and of the solutions of the model mixtures. Pharmaceutical grade metoprolol tartrate, metoprolol acid (M I) 2-hydroxy-3-(4-metoxyethylphenoxy) - propanoic acid (M II), α-hydroxymetoprolol (M III) and O-desmethylmetoprolol (H 105) were received from ASTRA Japan.

Solid - phase extractions of urine were carried out with SepPak C_{18} cartridges (Waters).

Standard Solutions and Samples

A stock solution of each compound was prepared by dissolving 10.0 mg of the compound in 100.0 ml of water. These solutions were stored in the dark at 4°C.

ITP Operating Systems

Cationic System

The leading electrolyte was 10 mM sodium acetate buffer (pH 4.0-5.5) - 0.1% methylhydroxyethylcellulose and the terminating electrolyte was 10 mM β- alanine. The driving current for the preseparation column was 200 μA and for the analytical column 40 μA (separation) and 10 μA (detection).

Anionic System

The leading electrolyte was 10 mM histidine-HCl (pH 6.0) - 0.1 % methylhydroxyethylcellulose and the terminating electrolyte was 5 mM morpholinoethanesulfonic acid. The driving current for the preseparation column was 250 μA and for the analytical column 40 μA (separation) and 10 μA (detection).

Sample Collection

Prior to dosing, blank urine was collected from each subject and a 10 - 15 ml sample frozen. Metoprolol (100 mg metoprolol tartrate, Vasocardin) was then administered orally to each fasting subject and the total urine collected for the 0-8, 8-16 and 16-24h periods. Samples (10-15 ml) of the measured collections were rapidly frozen and stored at -20°C until assay.

Preparation of the Urine Samples

Method A: Direct Injection

A control urine (1.0 ml) was spiked with a mixture of standards and 2 μl of the solution was injected.

Method B: Solid - Phase Extraction

A 0.1 ml volume of control urine was spiked with a solution containing accurately known amounts of compounds. A SepPak C_{18} cartridge was activated and conditioned by successive washing with 2 ml of methanol, 1 ml of distilled water and 1 ml of a solution containing 1 mM SDS in 10 mM phosphoric acid. A mixture of 0.1 ml of urine spiked with a mixture of standards was loaded on the cartridge. The urine samples pass through the cartridge at low pressure in 2-3 min. The substances were eluted with 2 ml of 25% acetonitrile in dichloromethane. The eluate was evaporated to dryness with a stream of nitrogen, the residue was dissolved in 1.0 ml of water and aliquot (50 - 100 μl) was injected into ITP.

Results and Discussion

These substances are such a heterogeneous group of compounds that there is no pH within operational range at which all of them carry a charge. Because of this, two consecutive runs were required to determine all compounds. Molecules containing the amino group (metoprolol, M I, M III and H 105) were separated at acidic pH as cations.

To find conditions giving a satisfactory separation the effect of pH of leading electrolyte on the separation of cations was studied. The pH was increased from 4.0 to 5.5 in steps of 0.25 units. In Fig.1 the relative step height values (r.s.h.s) are plotted versus the pH of 1 leading electrolyte. Relative step height was calculated using the equation $r.s.h = h_x / h_t$, where h_x is the height of the step of the compound and h_t is the height of the step of the terminating zone.The effective mobility of M I is not influenced by pH. In spite of the fact that M I has a low effective mobility in comparison with the effective mobility of the terminating ion, M I migrate in front of the terminating zone. The effective mobility $(u_{MI,T})_{eff}$ and consequently the electrophoretic velocity $v_{MI,T}$ of M I in the terminator zone is higher in comparison with the ITP velocity $(u_{MI,T})>u_{T,T}$; thus M I is enforced to migrate in front of the terminating zone. At pH higher than 5.5 the effective mobility of M I in the terminating zone is too low to achieve proper separation. Further experiments with other terminating electrolytes did not improve the separation of M I without decreasing the effective mobility of M I. Also, changing the ionic strength by varying the leading ion concentration did not significantly improve the separation. At pH 4.25 the effective mobilities of metoprolol, M III and H 105 are almost equal and these ions are not separable. Increasing the pH results in decreased r.s.h.s of metoprolol, M III and H 105. Increasing the pH has the greatest effect on H 105 step height, which decreases strongly. Thus the separation can be improved by increasing the pH of the

leading electrolyte. In general, the pH of the leading electrolyte is chosen in such a way that maximal differences in r.s.h.s can be obtained. Therefore, pH 5.0 was chosen.

Metabolite M II containing the carboxylic group was separated at pH 6.0 as anion. Relative step height value of M II at pH 6.0 equals 0.660.

Metabolite M I, containing both amino and carboxylic groups, in theory can be separated both as cation and as anion. If the pH of anionic leading electrolyte is above 9, M I has an effective mobility suitable for a separation according to the ITP principle. A disadvantage is that at such pH values disturbance from CO_2 from the air can be expected.

Fig. 2 illustrates the isotachopherograms obtained from extract of urine sample containing a mixture of five compounds each at a concentration of 50.0 µg/ml using the cationic (A) and anionic (B) system. The complete separation of metoprolol, M I, M II, M III and H 105 was achieved. No compounds present in urine interfered with the analytes.

Calibration

In order to determine the quantitative abilities of ITP separation, calibration graphs were set up for metoprolol, M I, M II, M III and H 105. In Table 1 the concentration ranges and the regression parameters are presented, showing a linear relationship between zone length and absolute amount injected. Five calibration points repeated three times were measured with control urine spiked with standards after solid-phase extraction.

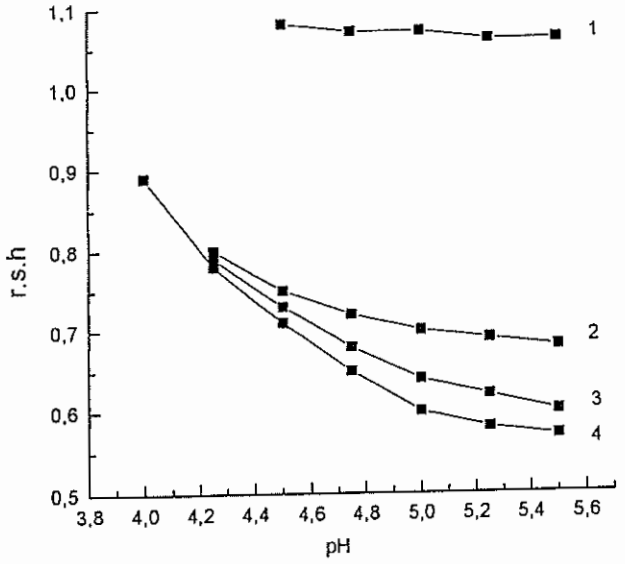


Fig 1. Effect of pH on relative step height (r.s.h). Leading electrolyte: 10 mM NaOH, acetic acid, pH 4.0 - 5.5. Terminating electrolyte: 10 mM β-alanine;
1- M I 2 - M III 3 - metoprolol 4 - H 105

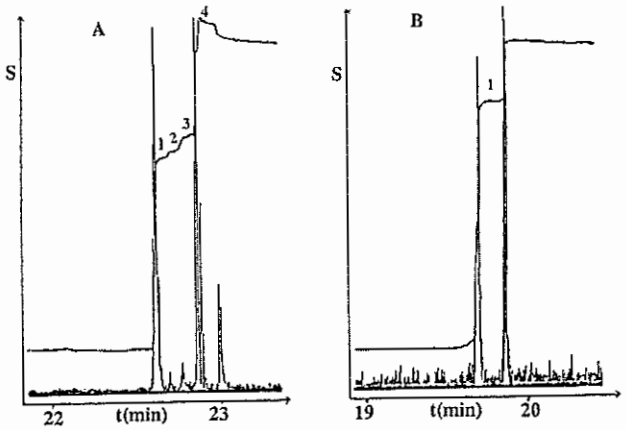


Fig 2. Isotachopherograms obtained from extract of urine sample containing (A) a standard mixture of H 105 (1), metoprolol (2), M III (3) and M I (4) in cationic system, and (B) standard of M II (1) in anionic system.
A: Leading electrolyte: 10 mM NaOH, acetic acid, pH 5.0, 0.1% m-HEC. Terminating electrolyte: 10 mM β- alanine
B: Leading electrolyte: 10 mM HCl, L-histidine, pH 6.0, 0.1% m-HEC TE: 5 mM MES

Table 1. Linearity of the method.

Compound	c(µg/ml)	a	b	r
Metoprolol	2.0-10.0	0.07	67.22	0.999
M I	10-50	0.06	65.96	0.999
M II	2-15	0.06	63.67	0.999
M III	5-15	0.05	71.23	0.999
H 105	1-5	0.04	64.46	0.999

The equation for the straight line is y=a+bx, where y is the zone length (mm), a is intercept on the ordinate, b is the slope (absolute amount injected, µg) and r is the correlation coefficient.

Table 2. Recovery (R) repeatability of the method in urine.

Compound	c(µg/ml)	R*(%)	RSD(%)
Metoprolol	1	84.6	3.4
	5	85.7	5.1
	10	85.2	4.8
M I	10	89.7	3.7
	15	89.4	4.8
	20	90.2	5.2
M II	1	89.7	4.4
	5	89.9	4.9
	10	87.8	3.8
M III	5	95.8	4.8
	10	98.2	3.9
	15	94.4	3.7
H 105	1	87.6	2.9
	2	88.3	3.6
	5	86.9	3.9

* Mean of five replications

Recovery and Repeatability

Aliquiots (0.1 ml) of urine were spiked with various amounts of the compounds. After the sample was extracted and analyzed as described, the zone length of each compound was compared with that obtained when the same amount of each compound in the absence of urine which had not undergone extraction was analyzed. Five replicate analyses were carried out at each concentration. The recovery of the compounds was studied by individual spiking, but all five compounds was studied were always included in the solution. The recovery and repeatability results are given in Table 2. The recovery varied from 84.6 to 95.8% and the relative standard deviation range from 2.9 to 5.2%.

Determination Limit

Table 3 shows the therapeutic level and the sensitivity of the procedure using the cationic and anionic system. Limit of determination is 0.5 μg/ml.

Real Samples

Method A

After investigation of 100 mg of metoprolol, two collections of urine (0-8h) and (8-16h), were analyzed by direct injecton of 2 μl of urine sample in cationic and anionic system.

A typical cationic isotachopherogram of the blank urine, the spiked urine and urine sample is shown in Fig. 3. Metoprolol, M I, M III and H 105 were well separated from urine compounds. Unfortunately, under the anionic condition used, metabolite M II and urine compound form a mixed zone together. Since therapeutic level of H 105 in urine is bellow the determination limit, only metoprolol and metabolites M I and M III were determined in real urine samples by direct injection.

The determination was achieved by the calibration and standard addition methods and statistically evaluated by linear regression. Seven calibration points were measured with standard solution (control urine spiked with compounds). Linearity was tested over the range 10 - 100 μg/ml. The correlation coefficients varied from 0.992 to 0.997. Recovery and repeatability were tested at three levels: 10, 50 and 100 μg/ml. The recoveries of metoprolol, M I and M III were nearly 100% with a standard deviation smaller than 3%. The injection volume may vary from 0.1 to 3.0 μl without any change in column efficiency. With an injection volume of 3.0 μl the limit of determination is 2 μg/ml.

Method B

Three collections of urine (0-8 h, 8-16 h and 16-24 h) were carried out after oral administration of 100 mg of metoprolol. Table 4 shows that ITP technique with SPE allows the determination of the concentration of metoprolol, M I, M II and M III in urine.

Conclusion

One of the advantages of ITP is the minimal sample pretreatment needed prior to analysis. In many cases, where the concentrations of the constituents in the sample are high enough, biological sample such as urine can be injected without isolation of the components prior to analysis. A good example of this is direct determination of metoprolol and its metabolites M I and M III in human urine.

Special problems can appear during direct analysis of urine, because low concentrations of the drug or their metabolites have to be determined in this matrice which contains high concentrations of other endogenous compounds. Samples that contain an excess of one

Table 3. Determination limits and therapeutic levels.

Compound	Determination limit (μg/ml)	Therapeutic level (μg/ml)
Metoprolol	0.5	0.2-40
H 105	0.5	<1
M III	0.5	3-20
M I	0.5	10-100
M II	0.6	1-15

Table 4. Determination of metoprolol and its metabolites M I, M II and M III in urine samples. Dose 100 mg metoprolol

Compound	c(μg/ml) 0-8 h	c(μg/ml) 8-16 h	c(μg/ml) 16-24 h
Sample 1			
Metoprolol	5.3±0.4	4.2±0.3	-
M I	48.1±0.3	31.7±0.5	10.4±0.3
M II	16.2±0.7	2.8±0.1	-
M III	12.7±0.5	6.0±0.4	5.5±0.4
Sample 1			
Metoprolol	6.1±0.5	4.9±0.5	-
M I	55.5±0.4	44.7±0.6	12.3±0.3
M II	15.8±0.8	3.1±0.4	-
M III	16.7±0.7	8.5±0.5	6.8±0.7

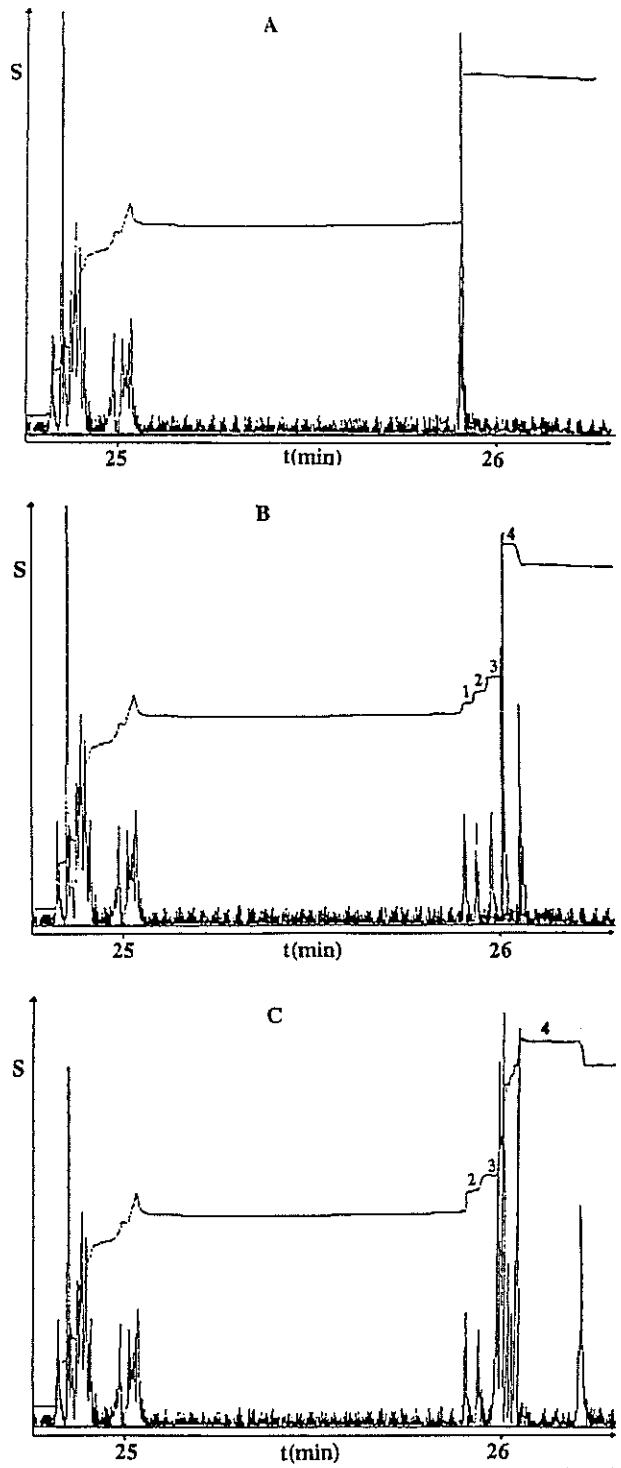


Fig 3. Cationic isotachopherograms of (A) metoprolol - free urine (B) urine spiked with metoprolol, M I, M III and H 105 and (C) urine collected over 0-8h from a patient dosed with 100 mg metoprolol tartate (Vasocardin)
1- H 105 2 - metoprolol 3 - M III 4- M I

ion with respect to another ion often make it difficult to resolve the excess concentration from the trace constituent. Dilution of the sample solves the problem of excess ions at the expense of lowering the concentration of the trace constituent below its detection limit. In ITP, considerations must be given not only to selectivity of the analytes, but to total ion capacity of the column. High concentrations of sodium and chloride, for instance, results in prolonged time of analysis and poor separation. Exessive amounts of sodium and chloride may be removed either by using column-coupling ITP or by sample preseparation. In most cases sample pretreatment procedures developed for other separation techniques (such as HPLC) may be applied.

The method of ITP determination of studied compounds is limited by the determination limit of ITP and the limited amount of sample, which can be prepared simultaneously. Despite a relatively high determination limit, ITP seems suitable for the determination of metoprolol and its metabolites M I, M II and M III in human urine. Although the sample pretreatment procedure is comparable with sample pretreatment prior to HPLC, the possibility of determining a spectrum of metabolites and low running costs make ITP a good alternative.

Clinically, metoprolol has been used with other β-blockers and diuretics. Some of these drugs have been tested with the assay to determine if they would interfere with the quantitation. Those tested were: labetalol, metipranolol, bisoprolol, amiloride, furosemide, chlorthalidone and hydrochlorothiazide. In each case, the zone for these drugs, if observed at all, did not interfere with those of metoprolol. Further tests with metabolites of these compounds will be done.

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