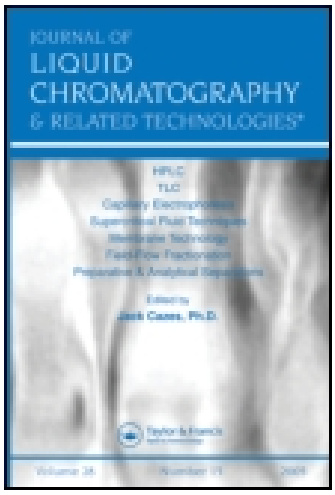

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A Combination of Isocratic and Gradient Elution Modes in HPLC with the Aid of Time-Overlapping Process for Rapid Determination of Methyldopa in Human Urine

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A new rapid time-overlapping high-performance liquid chromatography method using coupled-column double-injection technique with fluorescence detection has been developed and validated to determine methyldopa (MTD) in human urine. The method was based on injecting a new sample onto the second column before finalizing the cleanup and the re-equilibration of the first column for the former sample. A combination of isocratic and gradient elution was employed according to a pre-set program. At the beginning, isocratic step of acetate buffer solution (0.1 M, pH 2.4) was set until 7 min. Subsequently, a gradient elution step using acetate buffer (0.1 M, pH 2.4) as mobile phase A and acetonitrile as mobile phase B was employed. After the end of each gradient step, the column was re-equilibrated with 4 mL of the starting isocratic elution system before the next analysis. The overall cycle time was 7 min per each sample. The calibration curve was linear over the concentration range of 0.1–40 μg/mL MTD. The overall mean recoveries were in the range of 98.29–101.39%. The applicability of the method was successfully evaluated by monitoring the incremental urinary excretion of MTD in human urine over 12 hr after a single oral administration of 250 mg.

Keywords: coupled-column, double injection, fluorescence detection, HPLC, methyldopa, mixed elution modes

Introduction

Methyldopa (MTD) is an old antihypertensive agent, which is used in the treatment of mild to moderate hypertension. It is converted to α-methyl norepinephrine in adrenergic nerve terminals and its antihypertensive action appears to be due to the stimulation of the central α-adrenoreceptors by this agent.[1] Several analytical procedures have been reported for the analysis of MTD in pharmaceutical formulations or biological fluids. These procedures include determination by titrimetry,[2] spectrofluorimetry,[3] spectrophotometry,[4,5] potentiometry,[6] thin-layer chromatography,[7] gas liquid chromatography,[8] and high-performance liquid chromatography (HPLC)[9–23] methods. Furthermore, flow injection,[24] cyclic voltammetry,[25] nuclear magnetic resonance spectroscopy, and kinetic methods[26,27] have been reported. Electrochemical methods have been applied to determine MTD[28–34] due to their simplicity, accuracy, and rapidity.

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The urinary and the plasma concentrations of the drug have been most commonly measured by HPLC using electrochemical,[15,16,19–23] fluorescence,[10,12,14,20] UV,[9,13,14,17,18] or diode array[11] detections. Analytical conditions in the published methods consisted of gradient[10,14] or isocratic[9,11–13,15–23] elution of the mobile phase with[9,10,12,15,16,18–20,22,23] or without[11,13,14,17,21] using of an ion pair agent. Determination of MTD in biological matrices could not be performed without appropriate sample preparation, to remove potentially interfering components, even when using powerful analytical instruments, such as liquid chromatography–tandem mass spectrometry (LC–MS–MS).[35]

In liquid chromatography, the mobile phase should be selective for the components, and its composition is one of the most necessary variables influencing the separation. In isocratic elution, the mobile phase composition is unchanged during the separation. However, the disadvantages of an isocratic mode are broadening of the late-eluting peaks to the point of difficult detection, tailing peaks, and unnecessarily long separation times. Analysis time of complex mixtures with a wide range of retention factors can be made much shorter in the gradient elution chromatography, than in the isocratic separation of the same mixtures. However,

repetitive analyses using gradient elution need a long re-equilibration time to the initial conditions, which can extend the run time. For method development and optimization, reduction of the time needed for re-equilibration in the gradient elution is very important.

Until now only a few analytical HPLC methods for measuring urinary concentrations of MTD either individually or in combination with other drugs have been reported in the literatures.[15,17,20,22,23] The main problem in extracting low drug levels from complex samples, such as urine, is the limited concentration of the analyte available. MTD in these complex biological matrices is often present in low concentrations, along with extremely high background signals from the endogenous components, which interfere with the detection of the analyte. Sample preparation is a key step in the quantitative bio-analysis, and can potentially be a bottleneck in the process to develop robust and efficient methodologies. Combinations of off-line solid phase extraction (SPE) and cleanup procedures utilizing alumina and/or ion exchange resin have been described for the analysis of MTD in urine.[15,17,20,22,23] This combined extraction/cleanup strategy has drastically increased the time spent on sample handling and contributed significantly to the final cost of the analysis, both in terms of labor and consumption of materials. Minimization of error resulting from the human factor and the increasing demands for faster methods are major incentives to improve the classical procedures used for sample treatment of biological fluids. With a view to find an alternative method able to provide satisfactory results without the need for any sample pretreatment step, our study was involved in a research effort aimed to expand the automation by using a combined isocratic and gradient elution modes in HPLC together with the aid of time-overlapping process. This approach enabled us to directly inject and determine MTD in human urine samples.

The feasibility of the current method has been proven in conventional liquid chromatography using two gradient pumps, two identical ODS analytical columns and two identical injection valves. With a six-port-switching valve, human urine sample was loaded on one analytical column, while the second analytical column was re-equilibrated and conditioned for the next injection. Besides the opportunity of injecting urine sample directly to improve the precision, the developed method offered the possibility of removing the late-eluting endogenous interferences encountered in human urine in a short time. One of the most relevant aspects of this method was the time-overlapping process, i.e., injecting a new sample on the second analytical column before finalizing the cleanup and the re-equilibration of the first analytical column for the former sample. This latter capability was the key to success for enhancing sample throughput in the analysis of MTD in urine.

Experimental

Instrumentation

The chromatographic analysis was carried out in an HPLC system from Agilent (Agilent Technologies, CA, USA), equipped with two quaternary pumps, degassing devices, and two identical Rheodyne injectors with a 20 μL loop (Rheodyne, Berkeley, California, USA). A model Agilent 1200 Series G1321A fluorescence detector was used for the detection of MTD at an excitation wavelength of 270 nm and an emission wavelength of 320 nm. The chromatographic separation was achieved by means of two identical Thermo Scientific Hypersil ODS analytical columns (100 mm × 4.0 mm i.d., 5 μm), from Thermo Scientific (Florida, USA). A model 7010 flow direction column-switching valve was applied to facilitate the switching between the two analytical columns (Rheodyne, Berkeley, California). This instrument has a data station that controls its operating parameters, runs the desired programs, and records the detector signal. The measurements were done with the columns kept at room temperature (20°C ± 1°C).

General Procedure

The system setup for the rapid analysis consisted of two positions (A and B) (shown in Figure 1). Analyte was separated by reversed-phase HPLC using acetate buffer (0.1 M, pH 2.4) as mobile phase A, while acetonitrile as mobile phase B was used for achieving a complete column cleanup. In position A, preceding the injection of the urine sample, mobile phase A was brought onto the first analytical column by pump I at a flow rate of 1 mL/min for equilibration; meanwhile, pump II equilibrated the second analytical column with the same mobile phase at a flow rate of 1 mL/min. The urine sample (20 μL) was injected directly onto the first analytical column by pump I, and the analyte was separated from the endogenous components of the human urine matrix with an isocratic step consisting of 100% of mobile phase A. After 7 min, the switching valve was switched to position (B), and while MTD in urine sample was injected and chromatographed on the second analytical column, the first analytical column was cleaned-up with a linear gradient elution from 0% to 100% of mobile phase B within 0.5 min followed by an isocratic step consisting of 100% of mobile phase B for 2 min, and finally a linear gradient elution from 100% to 0% of mobile phase B within 0.5 min. The rest of the single run was continued by re-equilibrating the first analytical column with mobile phase A for 4 min to be ready for the next injection. After 7 min, the switching valve was switched back to its original position (position A), and while urine sample was injected and chromatographed on the first analytical column, the second analytical column was cleaned-up and re-equilibrated with the same gradient elution system. The overall cycle time was 14 min, including 7 min for column cleanup and re-equilibration between analyses, which can almost be halved in the case, where time-overlapping process was used, i.e., injecting a new sample on the second analytical column before finalizing the cleanup and the re-equilibration of the first analytical column for the former sample.

Materials and Reagents

MTD (99.87% purity) was obtained from ADWIC (Cairo, Egypt). The present method was applied to determine

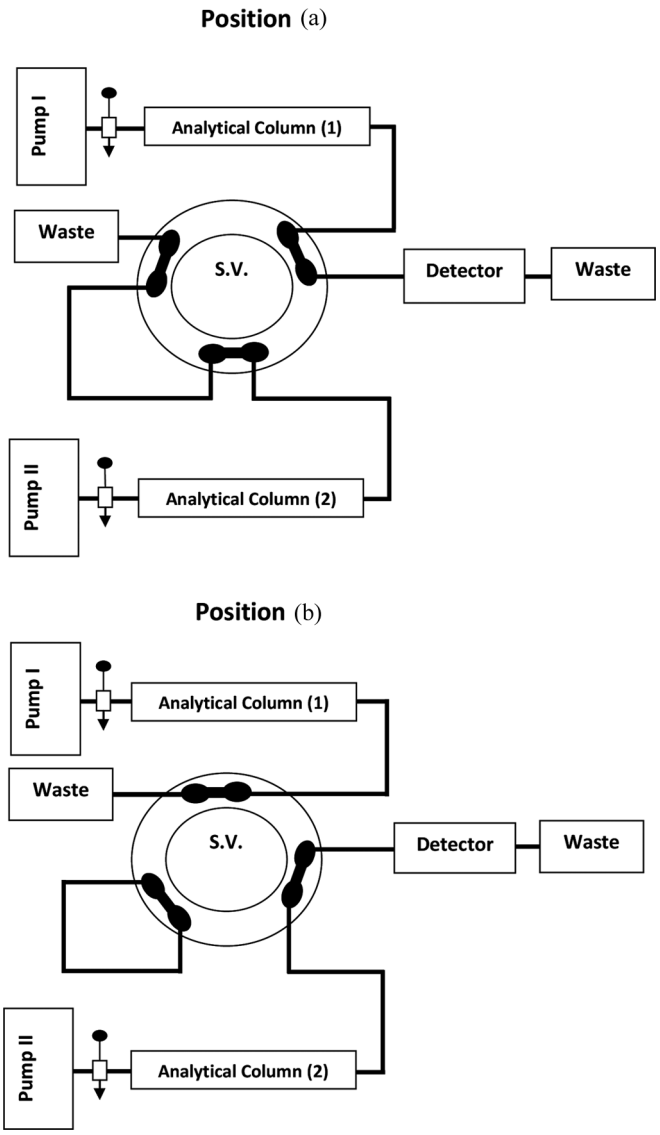

Fig. 1. Schematic diagram of the time-overlapping process for the rapid analysis of MTD in urine; the system in the initial position is ready for sample injection onto the first analytical column by pump I; meanwhile, pump II equilibrate the second analytical column (Position (a)); after 7 min, the switching valve is switched to Position (b), where a new sample is injected onto the second analytical column by pump II; meanwhile, pump I cleanup and re-equilibrate the first analytical column from the former sample. (S.V.: six-port-switching valve).

MTD in urine after the administration of Aldomet tablet: Aldomet tablet (Batch No. 01918) was manufactured by KAHIRA PHARM. & Chem. IND. CO., Cairo, Egypt. Each tablet was claimed to contain 250 mg of MTD. Acetonitrile used was HPLC grade (Sigma-Aldrich, Germany). Sodium acetate, orthophosphoric acid, and hydrochloric acid were of analytical grade (Sigma-Aldrich, Germany).

Calibration Standards and Quality Control Samples

A stock solution of MTD at the concentration of 1 mg/mL was prepared by dissolving appropriate amount of the drug in 0.01 M hydrochloric acid solution. Appropriate volumes of the stock solution of MTD were diluted with the same solvent to achieve the concentration ranges of 1–400 μg/mL. The standard solutions were protected from light by wrapping the containers with aluminum foil. Urine standards for calibration were freshly prepared. Each standard solution was diluted tenfold into drug-free human urine to obtain the concentration range of 0.1–40 μg/mL MTD. Calibration standards were stored at −20°C until required for assay. Prior to assay, frozen human urine samples were thawed at ambient temperature and centrifuged at 2000 g for 5 min at 4°C to precipitate solids followed by filtration of the supernatant through 0.45 μm Millipore filters to avoid the obstruction of the analytical column. An aliquot of 20 μL was injected onto the column for analysis. This precaution was important for successive analysis of urine samples without pressure trouble.

Quality control (QC) working solutions of MTD were prepared following the same procedure as that used for the preparation of MTD standard solutions. Specifically, the stock solution was further diluted to obtain three levels of QC standard working solutions (10, 100, and 300 μg/mL MTD). The QC standard working solutions were diluted tenfold into drug-free human urine to obtain the concentration range of 1–30 μg/mL MTD. The concentrations of the MTD QC samples were 1 μg/mL (low), 10 μg/mL (medium), and 30 μg/mL (high).

Recovery

Aliquots of 20 μL of the QC urine samples at three different concentration levels (1, 10, and 30 μg/mL MTD) were subjected to the described procedure. Five replicates of each QC sample were injected into the column. The recovery of the drug from the urine sample was assessed by comparing the peak area of MTD in urine sample to that of the aqueous solution with the same concentration of the analyte, and the assay recovery was calculated using the following equation:

$$\%\text{Recovery} = \frac{\text{mean measured concentration}}{\text{nominal concentration}} \times 100$$

Precision and Accuracy

Both precision and accuracy of the method were determined by analyzing five replicates of QC urine samples at low, medium, and high concentrations (1, 10, and 30 μg/mL MTD) against calibration curve. Intra-assay precision was calculated as the relative standard deviation (RSD%) of the mean concentration resulting from the same day. Inter-assay precision was assessed by the RSD% of the mean concentration on five consecutive days. The accuracy was determined by the percent of the relative errors (RE%) of the mean measured concentrations:

$$\text{RE}\% = \frac{\text{mean measured concentration} - \text{nominal concentration}}{\text{nominal concentration}} \times 100$$

Application

The validated method was applied to monitor the concentration of MTD in five selected male Egyptian volunteers (aged between 33 and 34 years and weighing between 93 and 95 kg) after a single oral dose of 250 mg MTD (Aldomet tablet, 250 mg/tablet). The volunteers have not taken any other medications for at least 2 weeks prior to the study. Prior to the drug administration, a 10 mL urine sample was taken as a control sample. Urine samples were collected (2, 4, 6, 8, and 12 hr after administration) in analytical variables dark-glass containers, and were immediately frozen at −20°C until analysis. Filtration of the thawed urine samples prior to injection is of great importance. Thawed samples often contain clots and solid particles, which on injection will give an immediate increase in the column backpressure owing to the restrictions of the inlet filter or the column packing. Frozen urine samples were first allowed to thaw at room temperature, centrifuged at 2000 g for 5 min at 4°C, and then filtered through 0.45 μm Millipore filters.

Results and Discussion

Optimization of the Elution Conditions

Different elution methods ranging from isocratic, gradient, and combination elution were tried to optimize the separation of MTD in human urine by HPLC. Initial efforts to develop an isocratic elution system for the separation of MTD using methanol-based mobile phase were unsuccessful and resulted in a poor resolution of the analyte from the endogenous matrix components. These efforts were subsequently shifted toward developing an elution system that would overcome the chromatographic limitations described above, and provide baseline separation of MTD. Two notable points emerged during the development of this system: (1) complete separation of MTD and the chromatographic quality were buffer concentration and pH-dependent, preferring acetate buffer to phosphate buffer; (2) this isocratic mode would require a long run time due to the presence of a series of strongly retained endogenous components in the human urine. It was observed that, a mobile phase consisted of acetate buffer (0.1 M, pH 2.4) gave an acceptable retention time (±SD) (5.80 min ± 0.047) of standard MTD with a good peak symmetry on conventional HPLC equipment using a Thermo Scientific Hypersil ODS analytical column (100 mm × 4.0 mm i.d., 5 μm). When this condition was used for further separation of MTD in untreated human urine, a longer run time was required for complete elution (37 min) (Figure 2a and 2b). This can be explained by the fact that, if the chromatographic conditions are adjusted for the satisfactory separation of the weakly retained compound (MTD), the elution of the strongly retained urine endogenous components will take very long time. On the other hand, if the chromatographic conditions are adjusted for the adequate retention of the strongly retained endogenous components of human urine, the weakly retained MTD will elute too early as a poorly separated band. When total gradient was employed, it possessed a disadvantage of requiring a time post-gradient to flush the initial mobile phase composition through the analytical column to ensure a reproducible retention time of the analyte in the subsequent injection. Since we were aiming to develop a method suitable for clinical studies, efforts were shifted to reduce the analysis time. In order to achieve this, a combination of isocratic and gradient elution was employed according to a pre-set program. We consider this gradient HPLC step, where, after the separation and the quantification of MTD (7 min), the solvent strength was increased in order to rapidly elute the late-eluting endogenous urine matrix components from the analytical column. At the beginning, the proportion of the acetate buffer solution (0.1 M, pH 2.4) was set at 100% until 7 min so that, the migration velocity of MTD along the analytical column was decreased, providing a sufficient separation of the early eluting MTD and the co-eluted endogenous components of the urine matrix. Subsequently, a gradient elution step using acetate buffer solution (0.1 M, pH 2.4) as mobile phase A and acetonitrile as mobile phase B was employed. The presence of acetonitrile in the gradient elution step was essential for the rapid cleanup of the analytical column, specifically, the elimination of the excessive late-eluting endogenous components of the urine matrix that were strongly retained under acetate buffer. After the end of each gradient step, the composition of the mobile phase was set back to the starting system, and the analytical column was re-equilibrated with 4 mL of the starting isocratic elution system (0.1 M acetate buffer, pH 2.4) before the next analysis. The composition of the mobile phase was changed according to the following time program: 0–7 min: 100% mobile phase A, 0% mobile phase B, isocratic; 7–7.5 min: linear gradient from 0% to 100% mobile phase B; 7.5–9.5 min: 100% mobile phase B, isocratic; 9.5–10 min: linear gradient from 100% to 0% mobile phase B; 10–14 min: 100% mobile phase A, isocratic.

The ultimate goal of the developed HPLC method was to obtain acceptable resolution of MTD within a reasonable analysis time. This goal has led to investigate the limits of speed in chromatography. In the present method, the analysis time was determined by the cycle time, which was the sum of the time required for the separation (i.e., isocratic development time) and the time required to cleanup and re-equilibrates the analytical column with the initial condition to prepare it for the next injection (i.e., cleanup and re-equilibration time). The time necessary for the column cleanup and re-equilibration increased the total run time of MTD in human urine, which should be as short as possible for maximum sample throughput. On the other hand, it should be long enough to reproducibly reestablish the equilibrium between the analytical column and the initial chromatographic isocratic condition. For an optimized separation (in terms of resolution), the isocratic development time should be considered fixed; therefore, the only way to minimize the cycle time was to decrease the cleanup and the re-equilibration time. In order to obtain a time analysis suitable for the routine use and to avoid the time required for complete cleanup and re-equilibration, we chose to

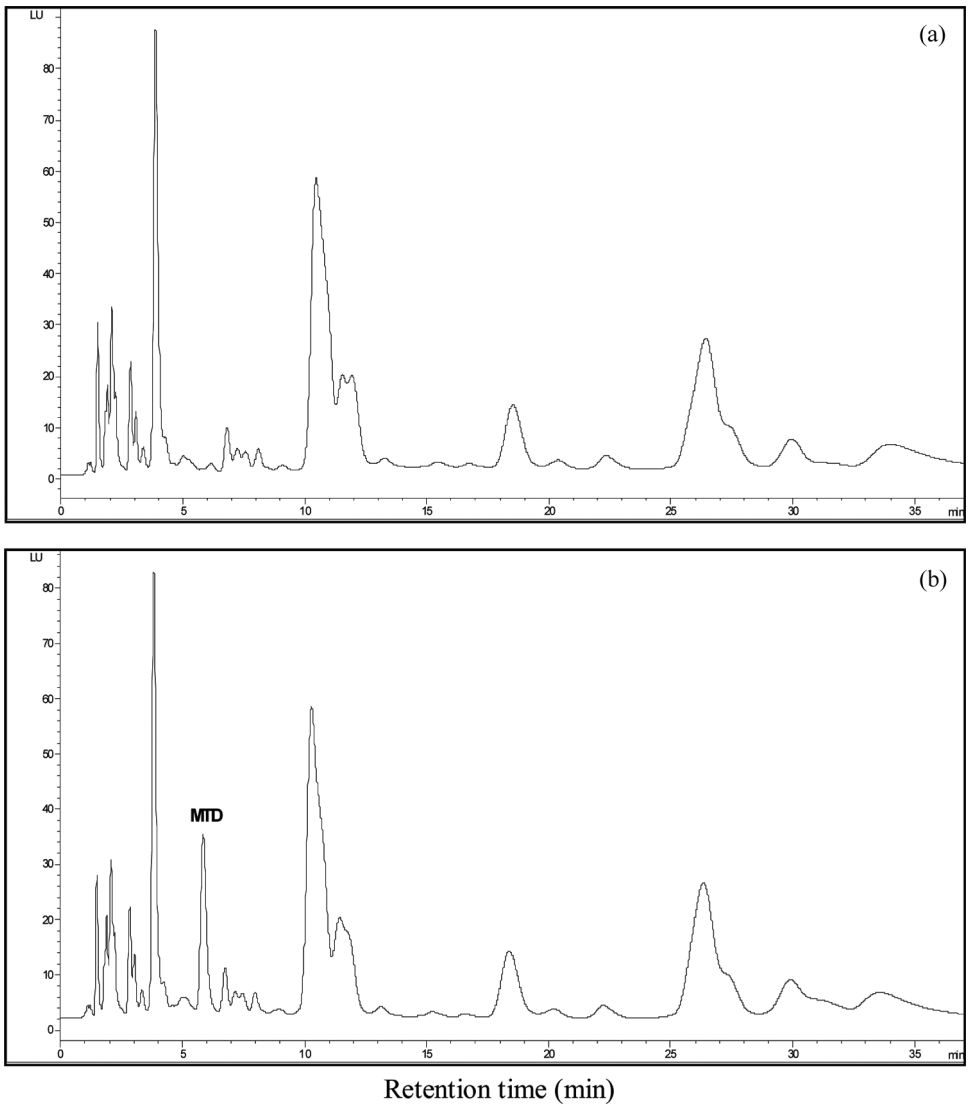

Fig. 2. Typical chromatograms obtained from the analysis of MTD in human urine using single column technique with fluorescence detection. (a) Drug-free human urine; (b) drug-free human urine spiked with MTD (10 μg/mL). MTD was separated and quantified on a Thermo Scientific Hypersil ODS analytical column (100 mm × 4.0 mm i.d., 5 μm) with a mobile phase consisting of acetate buffer (0.1 M, pH 2.4) at a flow rate of 1 mL/min.

modify the instrument by combining two analytical columns and two injection valves in single system manifold, called as couple-column double-injection technique. Such procedure required especially designed gradient elution HPLC instrumentation with a six-port-switching valve, two identical analytical columns, and two identical injection valves. This system allowed approximately two-times faster analysis than the single column technique of the same length at the same operating parameters. With a coupled-column double-injection technique and six-port-switching valve, human urine sample was loaded into one analytical column, while the late-eluting endogenous components of the previously urine sample were eluted and cleaned-up on the other analytical column. In other words, the overall cycle time was 14 min for the system, which could be almost halved in the case, where time-overlapping process was used, i.e., injecting a new sample on the second analytical column before finalizing the cleanup and the re-equilibration of the first analytical column for the former urine sample. Figure 3a and 3b shows chromatograms of directly injected urine sample by a Thermo Scientific Hypersil ODS (100 mm × 4.0 mm i.d., 5 μm) analytical column at flow rate of 1 mL/min resulted from typical blank urine (Figure 3a) as well as the spiked drug-free human urine sample chromatogram of 10 μg/mL MTD (Figure 3b). It can be clearly seen from these chromatograms that no interfering endogenous components of the untreated urine matrix were found in the retention time of MTD after 7 min isocratic (100% mobile phase A) procedure. Under this isocratic conditions, endogenous peaks were observed at 5.10 and 6.74 min; however, they did not disturb the actual detection of MTD. The reproducibility of the chromatographic conditions (retention time, peak

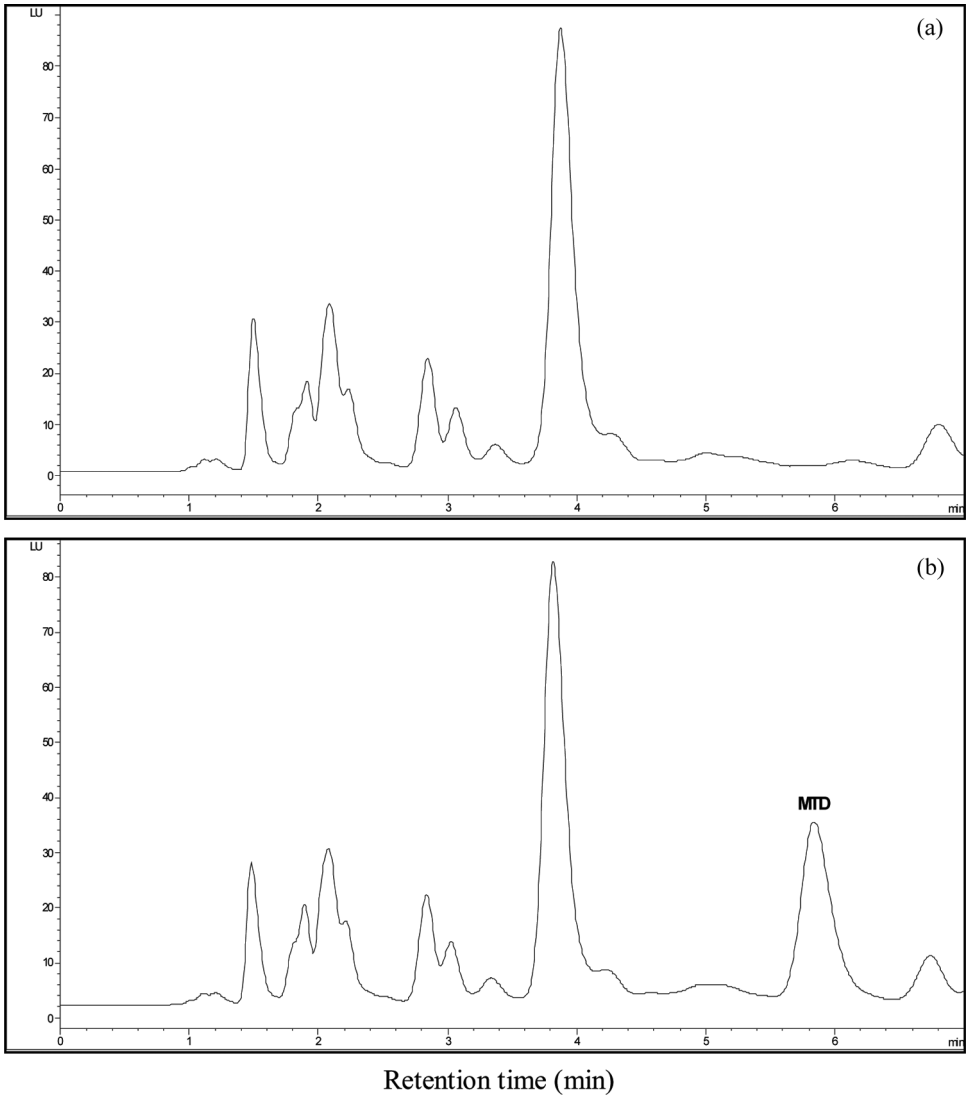

Fig. 3. Typical chromatograms obtained from the analysis of MTD in human urine using coupled-column double-injection technique (time-overlapping process) with fluorescence detection. (a) Drug-free human urine; (b) drug-free human urine spiked with MTD (10 μg/mL). MTD was separated and quantified on the first analytical column with an isocratic step consisting of acetate buffer (0.1 M, pH 2.4), at a flow rate of 1 mL/min. After 7 min, the switching valve was switched to the second column, and while urine sample was injected and chromatographed on this column, the first analytical column was cleaned-up and re-equilibrated with a binary gradient step using acetate buffer (0.1 M, pH 2.4) as eluent A and acetonitrile as eluent B to be ready for the next injection operation.

area) was determined by replicate injections (n = 5) of spiked drug-free urine samples with 10 μg/mL MTD. The standard deviation (SD) of the peak areas of MTD was found to be ±0.895 and the mean retention time for MTD ±SD was 5.80 ± 0.047 min.

Selection of Time-Events for Column-Switching

The time-overlapping process does not have to wait until complete column cleanup and re-equilibration of the first analytical column to reintroduce the new urine sample into the second analytical column. In fact, the next injection process can be initiated on one analytical column when the cleanup stage begins on the other analytical column. In the initial position, position A (Figure 1); human urine sample was loaded onto the first analytical column. The switch to position B should occur when the MTD peak in the untreated human urine has been eluted and detected. It has been shown that, MTD in urine could be eluted within 5.80 min for a Thermo Scientific Hypersil ODS analytical column (100 mm × 4.0 mm i.d., 5 μm) using acetate buffer (0.1 M, pH 2.4). In order to avoid the overlapping of the highly retained endogenous components of the urine matrix from the first run with MTD from the second run, the time point to inject the next sample must be adjusted so that, a complete cleanup of the late-eluting endogenous components of the urine matrix was achieved. All late-eluted endogenous components of the urine matrix were cleaned-up

in less than 3 min when eluted with a binary gradient elution system of mobile phase A (0.1 M acetate buffer, pH 2.4) and mobile phase B (acetonitrile). The six-port valve was therefore kept in position B for 7 min before switching back to position A to achieve complete column cleanup and re-equilibration with mobile phase A (4 min) prior to the next sample injection. Since separation on the first analytical column, as well as, complete column cleanup and re-equilibration of the second analytical column can take place simultaneously, a total analysis time of 7 min for each sample could be achieved.

Merits of the Time-Overlapping Process

In this work, the application of the time-overlapping process HPLC method has been established for the first time to determine MTD. Overlapping development stages, combined with fluorescence detection, is regarded as a core technique for faster analysis of MTD in human urine. Besides providing a higher resolution and a shorter separation time, the time-overlapping process has a characteristic advantage over other conventional gradient HPLC methods in that; the final stage of cleanup prepares the analytical column for re-equilibration to begin the next injection operation. With this in mind, repeated separation–cleanup and re-equilibration can be performed. In this case, the injection of a new sample can occur before the previous analytical column has been cleaned-up and re-equilibrated. Figure 1a and 1b shows typical repeated separation–cleanup and re-equilibration operations, where a urine sample can be injected onto the second analytical column before finalization of the cleanup and the re-equilibration of the first analytical column for the former sample.

Method Validation

Linearity

To determine the linearity of the HPLC detector response, calibration standard solutions for MTD were prepared as described in the text. The calibration curve from directly injected spiked urine samples was constructed by plotting the measured peak areas against the concentrations of the drug in the concentration range of 0.1–40 μg/mL. Sample concentrations were determined by linear regression, using the formula $Y = a + bC$, where Y = peak area, C = concentration of the standard in μg/mL, b = the slope of the curve, and a = the intercept with Y-axis. Characteristic parameters of the linear calibration curve are shown in Table 1.

Limit of Detection and Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) were determined according to the ICH guidelines for validation of analytical procedures[36] and were found to be 0.0296 μg/mL and 0.0896 μg/mL, respectively (Table 1).

Recovery, Precision, and Accuracy

Recoveries for MTD in human urine were found to be 98.91%, 98.46%, and 101.39% for QC samples at low (1 μg/mL), medium (10 μg/mL), and high (30 μg/mL) concentration levels, respectively. In order to judge the quality of the elaborated method, precision and accuracy were validated on the basis of intra- and inter-assays. A summary of intra- and inter-day precisions and accuracies at QC concentrations is shown in Table 2. At three different concentrations, the intra-day reproducibility and accuracy for the urine samples were excellent, with RSD% being in the range of 0.22–0.97% and with mean RE% ranging from 1.39% to −1.54%. At the same concentration levels, the inter-day RSD% was in the range of 0.20–0.91% and the mean RE% ranged from 0.27% to −1.71%. Repeatability and reproducibility of MTD in urine samples with high and low concentration levels were below the value of 1.80%, indicating a reliable measurement using the proposed method (Table 2).

Table 1. Characteristic parameters for the regression equations of the proposed method

Parameters	MTD
Calibration range (μg/mL)	0.1–40
Detection limit (μg/mL)	0.0296
Quantitation limit (μg/mL)	0.0896
Slope (b)	41.1752
Standard error of the slope	0.2831
Intercept (a)	2.8932
Standard error of the intercept	5.5056
Correlation coefficient (r^2)	0.9997

$Y = a + bC$, where C is the concentration of MTD in μg/mL and Y is the peak area.

Selectivity and Application

The selectivity was investigated by preparing and analyzing five individual human blank urine samples and samples of drug-free human urine spiked with MTD (10 μg/mL). Each urine sample was tested using the described procedure. Representative chromatograms of blank human urine sample, and sample spiked with MTD (10 μg/mL) are shown in Figure 3a and 3b. Good selectivity for the analyte was obtained as evidenced by the symmetrical resolution of the peak. There was no significant chromatographic interference close to the retention time of the analyte in the untreated human urine samples. The typical retention time ±SD for MTD was 5.80 ± 0.047 min. The total run time was about 7 min.

Table 2. Precision and accuracy validation of MTD

	Concentration (μg/mL)		
	Nominal	Mean recovery[a] (% ±RSD)	Mean RE (%)
Intra-assay[a]	1	98.91 ± 0.97	−1.09
	10	98.46 ± 0.22	−1.54
	30	101.39 ± 0.36	1.39
Inter-assay[a]	1	98.49 ± 0.91	−1.51
	10	98.29 ± 0.20	−1.71
	30	100.27 ± 0.63	0.27

[a]Average of five determinations.

A high degree of confidence in the validity of the new time-overlapping HPLC method was showed in the suitability of this technique to monitor the incremental urinary excretion of MTD over 12 hr in five selected male Egyptian volunteers (aged between 33 and 34 years and weighing between 93 and 95 kg) after a single oral dose of 250 mg MTD (Aldomet tablet, 250 mg/tablet), as there were no potential concomitant interferences arising from the matrices. Figure 4a and 4b shows chromatograms of a blank urine sample taken from the volunteer before administrating the drug (Figure 4a), as well as the chromatogram of the clinical urine sample collected after 8 hr from orally administering 250 mg MTD (Figure 4b). A summary of the urinary concentrations of MTD (μg/mL) at 2, 4, 6, 8, and 12 hr after a single oral dose of 250 mg MTD is shown in Table 3. It was clearly observed that, urinary concentrations of MTD showed degrees of variability within and between subjects in these preliminary experiments. Also, the maximum urinary concentration of unchanged MTD was recovered, in all volunteers, when urine samples were collected 6 hr after a single oral dose. The high similarity between age, weight, and sex of the selected Egyptian volunteers can explain these results.

The proposed coupled-column double-injection strategy for the determination of MTD in human urine is superior to other published HPLC methods with respect to simplicity, rapidity, and sensitivity. The linearity range was the parameter used to compare the sensitivity of the proposed method with the other reported methods. The reported linearity ranges (μg/mL) of MTD were found to be 5–170[17] and 2–500.[23] In the developed method, the calibration curve showed excellent linearity with correlation coefficient of 0.9997 over the range of 0.1–40 μg/mL. On the other hand, intensive sample cleanup and enrichment by off-line SPE and liquid–liquid extraction were necessary to achieve the required selectivity and sensitivity in the reported methods.[15,17,20,22,23] The sample cleanup procedures limited the ultimate performance of these methods, especially with

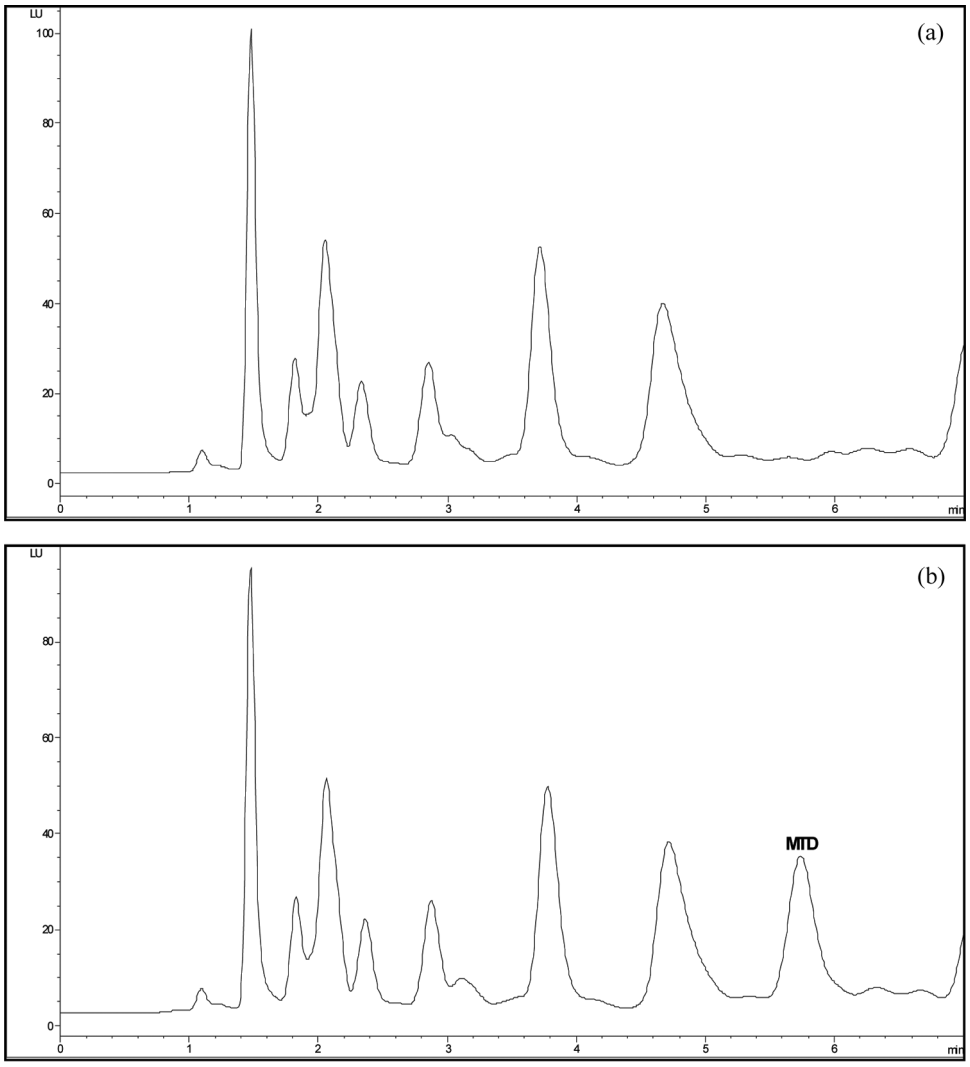

Fig. 4. Chromatograms of typical drug-free human urine sample (a); and clinical urine sample obtained at 8 hr after a single oral dose of 250 mg MTD (b).

Table 3. Incremental urinary excretion of MTD in five selected male Egyptian volunteers

	Urinary concentration of MTD (μg/mL) after a single oral dose of 250 mgMTD(Aldomet 250 mg) obtained at the following time intervals				
Volunteer	2 hr	4 hr	6 hr	8 hr	12 hr
1	4.37	12.24	14.24	9.70	8.54
2	8.53	15.33	17.17	13.32	10.04
3	4.47	12.71	14.42	10.06	8.73
4	3.47	8.63	11.25	8.01	7.34
5	8.46	14.74	16.66	12.44	9.67
Mean ± SD	5.86 ± 2.44	12.73 ± 2.64	14.75 ± 2.35	10.71 ± 2.15	8.86 ± 1.06

regard to ruggedness and reliability. Whereas, the implementation of the combined modes of isocratic and gradient elution with the aid of time-overlapping process can utilize direct injection procedure. Therefore, current method is less time consuming, and uses small amounts of organic solvents than other published methods, besides, the possibility of human error arising during the several pretreatment steps is considerably reduced. In addition, the current method provides a precise methodology that overcomes any interference from the endogenous matrix components, and since the recovery of MTD is quantitative, the internal standard could be safely eliminated.

Stability

The stability studies of MTD in urine were carried out to ensure the reliability of the results in relation to handling and storing of the urine samples. The studies involve evaluating the freeze and thaw stability, short-term stability, and long-term stability. The tests of stability were assessed with two concentrations of QC samples, i.e., 1 and 10 μg/mL. In the freeze and thaw stability test, the samples were stored at −20°C for 24 hr and thawed at room temperature for approximately an hour. Triplicate analyses of the samples at each concentration were quantitated. Samples were immediately refrozen at −20°C for the next study day. This cycle was repeated for three consecutive days. The short-term stability was assessed after the storage of the samples at 5°C and at room temperature. Carrying out the experiment after the storage of the samples at −20°C for 4 weeks assessed the long-term stability. The concentration of MTD after each storage period was related to the initial concentration as determined for the samples that were freshly prepared. Experiments showed that there was no difference in the mean peak areas after one, two, and three freeze-thaw cycles and freshly prepared urine samples. Also, MTD in urine samples exhibited no chromatographic changes when stored refrigerated at 5°C for 3 days, and at −20°C for 4 weeks. Urine samples were found to be stable at room temperature upon standing for at least 8 hr.

Conclusion

A new approach for high-throughput monitoring of MTD in human urine has been developed by modifying a conventional gradient elution HPLC. Most importantly, the novel instrument configuration substantially reduces the time needed to re-equilibrate the analytical columns between consecutive gradient runs, thereby reducing the total time for each analysis. The overall cycle time was 14 min for the single column analysis, which could be halved in the case time, where time-overlapping process was used, i.e., injecting a new sample on the second analytical column before finalizing the cleanup and the re-equilibration of the first analytical column for the former sample. No interference in the assay from any endogenous components or other concurrently used drugs was observed. The reduced sample handling and the short run time made it possible to analyze eight samples per hour. Validity of the method was studied and the method was precise and accurate within a linearity range from 0.1 to 40 μg/mL. The high sensitivity and selectivity of the coupled-column double-injection procedure, makes it a suitable technique for the analysis of MTD in human urine samples.

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