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Article

An Eco-Friendly Direct Injection HPLC Method for Methyldopa Determination in Serum by Mixed-Mode Chromatography Using a Single Protein-Coated Column

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A simple, rapid and environment-friendly direct injection HPLC method for the determination of methyldopa (MTD) in human serum has been developed and validated. The method was based on cleanup and separation of MTD from serum by mixed-mode liquid chromatography using a single protein-coated TSK gel ODS-80 TM analytical column (50 × 4.0 mm i.d., 5 μm). The protein-coated column exhibited excellent resolution, selectivity and functioned in two chromatographic modes: size-exclusion chromatography [i.e., solid-phase extraction (SPE) for serum proteins] and reversed-phase chromatography for the final separation of MTD. SPE and HPLC separation were carried out simultaneously with a green mobile phase consisting of acetate buffer (0.1 M, pH 2.4) at a flow rate of 1 mL/min and at room temperature (23 ± 1°C). The eluent was monitored at emission and excitation wavelengths of 320 and 270 nm, respectively. A calibration curve was linear over the range of 0.1–30 μg/mL with a detection limit of 0.027 μg/mL. This online SPE method was successfully applied to real samples obtained from patients receiving MTD therapy.

Introduction

Methyldopa (MTD; 3-hydroxy-α-methyl-L-tyrosine sesquihydrate) 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 HPLC (9–23) methods. Furthermore, flow injection (24), cyclic voltametry (25), nuclear magnetic resonance spectroscopy and kinetic methods (26, 27) have been reported.

MTD has been most commonly measured in biological fluids 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. The 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) (28). The isolation and the quantification of MTD from biological samples using HPLC present many challenges. The major challenge is the removal of macromolecules (i.e., proteins), to avoid damage to the chromatographic column. Protein in the biological fluids can be precipitated or denaturated and adsorbed onto the packing material, leading to backpressure buildup, change in retention time and decreased column efficiency and capacity. Protein denaturation and precipitation occur as a result of the high concentration of the organic solvents present in the mobile phase used for the elution of drugs from analytical columns. Therefore, a given set of more or less complex steps is required for sample preparation before injection into the chromatographic system. Conventionally, sample preparation has been performed by means of protein precipitation (PP), liquid–liquid extraction or off-line solid-phase extraction (SPE). Prior to chromatography, pre-purification steps are usually performed employing PP (10, 11, 14, 15, 21) and off-line SPE (12, 16). The cleanup procedures often take up the majority of the total analysis time and contributed significantly to the final cost of the analysis, both in terms of labor and the consumption of materials. Also, traditional HPLC methods using conventional sample preparation steps require significant amounts of organic solvents and chemicals, which are dangerous not only to the analyst, but also to the environment. With growing awareness about the environment, the development of green methodologies has been receiving increasing attention.

Modern trends in sample preparation have focused on the development of automated extraction procedures by online SPE coupling with the analytical column to reduce the sample volume required, the analytical time and the solvent consumption. Column-switching chromatography is a valuable tool in analytical chemistry as it can combine the automation capabilities of online SPE technique and the separation power of HPLC (29). An initial attempt to establish a rapid HPLC method was the direct injection of the serum sample onto small pre-columns packed with conventional reversed-phase materials. However, problems associated with the tight adsorption of some proteins to the pre-column and the rapid pressure buildup at the head of the column due to protein denaturation were observed. To submit untreated serum into the system and improve the performance and lifetime of the pre-column, integration of a protein-coated pre-column in an online injection system appeared as an attractive approach (29–31). Compared with conventional reversed-phase silica

pre-columns, the protein-coated pre-column has attracted significant interest because of its ease of preparation, high reproducibility and rapid mass transport. We have developed direct injection procedures for the determination of various drugs in human plasma and serum using the column-switching technique and protein-coated pre-columns together with analytical columns (29–31).

The protein-coated TSK gel ODS-80 TM silica column is characterized in that it has non-adsorptive outer particle surfaces (size-exclusion chromatography) towards macromolecular matrix components (e.g., proteins) that will pass through column unimpeded, while low-molecular molecules (drugs) have free access to the binding centers and thus can be selectively retained (32). In other words, serum proteins and macromolecules could not be adsorbed further on the external surface of the protein-coated column, and they could not enter into the interior of the small pores, thus they flowed out of the extraction column regardless their polarities.

We thought it might be convenient to determine drugs in biological fluids by a single-column method to simplify our laboratory procedures. There has been enormous interest in rapid separations in laboratories that analyze large numbers of biological fluid samples per day. Therefore, we focused mainly on the following requirements: speed to achieve high sample throughput, reliability and linearity over a large concentration range to cover the concentration levels from the therapeutic MTD use. For this purpose, we combined HPLC online SPE and separation in a single protein-coated column with fluorescence detection. The protein-coated analytical column approach enabled us to extract and separate the MTD from the sample matrix in a very short time (<4 min).

Experimental

Instrumentation

The HPLC separation and quantitation were carried out on a homemade protein-coated TSK gel ODS-80 TM (50 × 4.0 mm i.d., 5 μm particle size) analytical column from Tosoh Corporation (Tokyo, Japan). The apparatus consisted of solvent delivery pump (Agilent 1200 Series Iso pump G1310A, Agilent Technologies, CA, USA). A model 7725i sample injection valve (20 μL) was applied to load the sample onto the analytical column (Rheodyne, Berkeley, CA, USA). A fluorescence detector (Agilent 1200 series, G1321A) was used for the detection of MTD at emission and excitation wavelengths of 320 and 270 nm, respectively. Data acquisition was performed on Agilent LC ChemStation. All experiments were carried out at ambient temperature (23 ± 1°C).

Materials and reagents

MTD (99.87% purity) was obtained from ADWIC (Cairo, Egypt). The present method was applied to determine MTD in human serum 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. Bovine serum albumin (BSA) of fraction V powder was obtained from Sigma-Aldrich, Germany. Rabbit dialyzed plasma (plasma protein fraction) was prepared by three times dialysis of rabbit plasma in visking tube against 100 volumes of phosphate-buffered saline, pH 7.4, at 25°C for 4 h. Methanol used was HPLC grade (Sigma-Aldrich, Germany). Potassium dihydrogen phosphate, sodium acetate, orthophosphoric acid, hydrochloric acid, sodium chloride, potassium chloride, disodium hydrogen phosphate, ethylenediamine tetraacetic acid disodium salt, sodium hydroxide and chloroform were of analytical grades (Sigma-Aldrich, Germany). TSK gel ODS-80 TM silica (5 μm) was from Tosoh Corporation. Distilled water was used for the preparation of all reagents and solutions.

Phosphate-buffered saline, pH 7.4

The phosphate-buffered saline stock solution contains 80 g sodium chloride, 2 g potassium chloride, 11.5 g disodium hydrogen phosphate, 2 g potassium dihydrogen phosphate and 20 mL of 0.1 M ethylenediaminetetraacetic acid disodium salt per liter. The pH was adjusted to 7.4 with 1 M sodium hydroxide solution.

Mobile phase

A mobile phase consisting of acetate buffer (0.1 M, pH 2.4) was employed in the assay procedure to separate the drug from the endogenous components of the human serum. The mobile phase was freshly prepared on the day of use, filtered through 0.45 μm filters (Millipore, Billerica, MA, USA) and degassed ultrasonically under vacuum.

Column preparation

In this study, a dual-purpose (online SPE and chromatographic separation) strategy was introduced for the determination of MTD from human serum by using a protein-coated TSK gel ODS-80 TM (5 μm) analytical column. The packing materials (TSK gel ODS-80 TM) were suspended in chloroform, degassed under vacuum with continuous stirring for 10 min. A stainless-steel cylinder (100 × 7.5 mm i.d.) was used as a reservoir for the packing materials. This reservoir was connected to a short column (50 × 4.0 mm i.d.) and the suspended ODS-80 TM supplied from the reservoir was packed into the column with the aid of an HPLC pump at a flow rate of 5 mL/min with methanol as a purge solvent (10 min). Pumping must continue until a constant pressure is reached. The cylinder was then disconnected and a mixture of methanol and distilled water (1 : 1) was passed through the column at a flow rate of 1 mL/min for further 10 min.

The TSK gel ODS-80 TM analytical column was equilibrated with phosphate buffer saline, pH 7.4. Then, 20 μL of 6% BSA solution was injected at a flow rate of 1 mL/min and at ambient temperature (23 ± 1°C). The column was then equilibrated with 0.1 M phosphate buffer solution, pH 3.0, and again 20 μL of 6% BSA was injected. The column was then washed with methanol. The above procedures (BSA saturation and methanol washing) were repeated several times. The column thus obtained is called BSA-coated analytical column. The protein-coated analytical column was prepared from the BSA-coated analytical column as follows: 20 μL of the rabbit dialyzed plasma was injected at a flow rate of 1 mL/min with phosphate buffer saline, pH 7.4, at ambient temperature. The column was then equilibrated with 0.1 M phosphate solution, pH 3.0, and again 20 μL of the rabbit dialyzed plasma was injected. The column was then washed with

methanol. These steps were important to immobilize the absorbed proteins on the outside surface of the porous packing materials. The interaction of BSA with plasma proteins may be important for both protein immobilization and also to lose the affinity for natural plasma proteins. Thus, the above procedures (plasma protein saturation and methanol washing) were repeated several times.

Standard solutions and quality control samples

A stock standard solution of MTD (1 mg/mL) was prepared by dissolving an accurately weighed amount of MTD (100 mg) in 100 mL of 0.01 M HCl solution. Appropriate volumes of the stock standard solution of MTD were diluted with the same solvent to prepare working solutions of MTD over the concentration range of 1–300 μg/mL. The standard working solutions were protected from light by wrapping the containers with aluminum foil. Serum standards for calibration were freshly prepared. Each standard working solution was diluted 10-fold into drug-free human serum to obtain the concentration range of 0.1–30 μg/mL of MTD. Calibration standards were prepared in bulk and dispensed in 1 mL of aliquots into properly labeled Eppendorf tubes and stored at −20°C until required for assay. Prior to assay, frozen human serum samples were thawed at ambient temperature, centrifuged at 4000 rpm for 10 min and filtered through 0.45 μm disposable Millipore filters to avoid the obstruction of the protein-coated analytical column (Agilent premium syringe filters with mini-tip regenerated cellulose polypropylene, 13 mm diameter). These precautions were important for successive analysis of serum samples without pressure trouble. An aliquot of 20 μL was injected onto the column for analysis.

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

General procedure

A 20-μL aliquot of human serum was injected directly onto the protein-coated analytical column (50 × 4.0 mm i.d., 5 μm particle size) with a green mobile phase consisting of acetate buffer (0.1 M, pH 2.4), at a flow rate of 1 mL/min and at room temperature (23 ± 1°C). The eluent was monitored at emission and excitation wavelengths of 320 and 270 nm, respectively.

Extraction recovery, precision and accuracy

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

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

Both precision and accuracy of the method were determined by analyzing five replicates of QC serum samples at low, medium and high concentrations (1, 10, and 30 μg/mL of MTD) against a 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 error (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 500 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, 10-mL venous blood samples were taken as a control sample. Venous blood samples (10 mL) were collected at 3 h following the administration. Blood samples were centrifuged at 4000 rpm for 10 min and the serum was taken and stored at −20°C until analysis.

Results

In the proposed HPLC procedure, the untreated human serum sample is directly injected into a liquid mobile phase. The mobile phase carries the sample through the protein-coated column that separates sample's components based on their ability to partition between the mobile phase and the stationary phase. For the choice of a mobile phase system for the protein-coated column, it was essential to have the possibility of regulating the retention of the MTD and to separate it from the serum endogenous matrix components. Also, the mobile phase should be compatible with the serum, i.e., serum proteins should be kept in solution. In this study, a short protein-coated ODS-80 TM (50 × 4.0 mm i.d., 5 μm) analytical column was used for the cleanup and the separation of the drug from the serum sample with an aqueous solution of buffers. To verify the applicability of these buffers for the chromatographic determination of MTD in serum, selections of the pH as well as the concentration of the buffer used as the mobile phase were studied thoroughly to optimize the analytical conditions. The effect of pH parameter was studied in the pH range of 2.2–3.5 using buffer solutions from phosphate and acetate. Acetate buffer gave the best performance and was selected in all further experiments. Variation in the pH values strongly affected the retention behavior of MTD. It was found that, acetate buffer mobile phase of pH 2.4 gave a very good resolution between the analyte and the endogenous components of the serum matrix. The variation in the retention time of MTD was

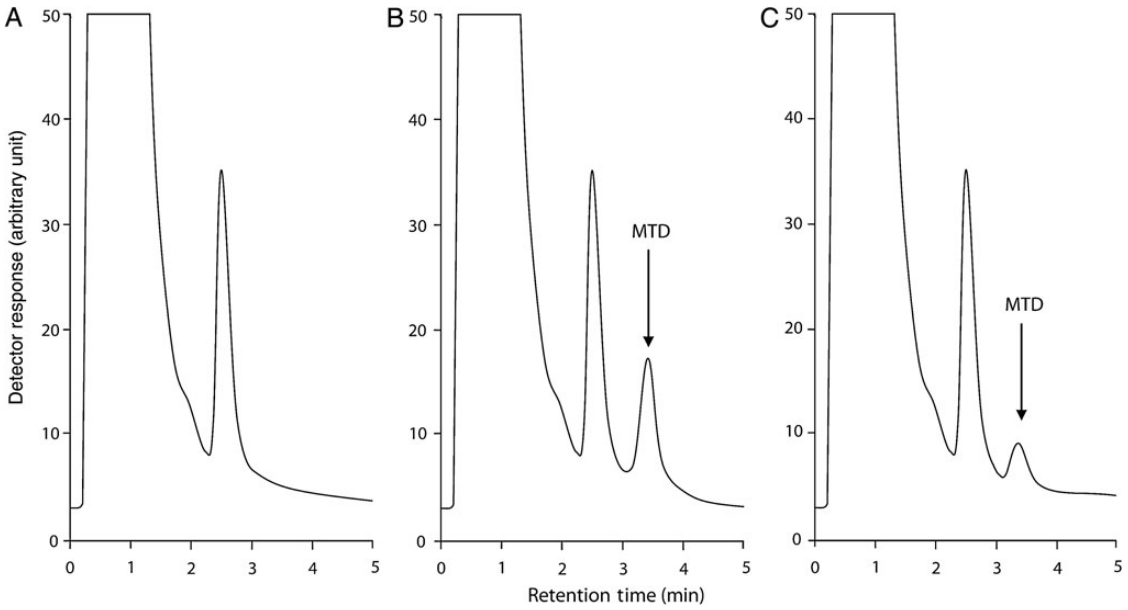

Figure 1. Typical chromatograms obtained from the analysis of MTD in human serum by mixed-mode liquid chromatography using a single protein-coated analytical column with fluorescence detection. (A) Drug-free human serum; (B) drug-free human serum spiked with MTD (10 μg/mL) and (C) clinical serum sample obtained at 3 h after a single oral dose of 500 mg MTD.

also examined using acetate buffer of concentrations varying from 0.05 to 0.2 M. Acetate buffer (0.1 M) showed significant improvement in the baseline resolution and was selected as the optimum concentration. From series of tests, the separation and the detection were achieved by eluting MTD with the examined mobile phase at a flow rate of 1 mL/min, and by monitoring the eluent at emission and excitation wavelengths of 320 and 270 nm, respectively. The detection wavelengths were chosen using the spectrum mode of the fluorescence detection with respect to the maximum sample signals. This mode enabled the determination of the optimum emission and excitation wavelengths in real conditions during measurement. Using a protein-coated ODS-80 TM analytical column (50 × 4.0 mm i.d., 5 μm), a mobile phase of acetate buffer (0.1 M, pH 2.4) was found to give acceptable separation in a short time. Accordingly, faster separation was possible and the productivity of the chromatographic processes was increased. Figure 1A and B shows chromatograms of a directly injected serum sample to a single protein-coated TSK gel ODS-80 TM analytical column (50 × 4.0 mm i.d., 5 μm) at a flow rate of 1 mL/min resulted from a typical blank serum (Figure 1A), as well as a spiked drug-free human serum sample chromatogram of 10 μg/mL of MTD (Figure 1B). It can be clearly seen from these chromatograms that no interfering endogenous components of the serum matrix were found in the retention time of MTD, and the MTD peak was well separated within 4 min.

Method validation

Linearity, limit of detection and limit of quantification

The calibration range was established in consideration of the practical range necessary according to the MTD concentrations after administration of the therapeutic doses. The calibration curve from directly injected spiked serum samples was constructed by plotting the measured peak area versus the concentrations of MTD over the concentration range from 0.1 to 30 μg/mL. Each concentration was repeated three times; this approach provided information on the variation in peak area values between samples of same concentration. The linearity of the calibration curve of MTD was validated by the high value of the regression coefficient (0.9996). The coefficient for the linear equation $Y = a + bC$ was calculated using the linear regression least squares method, where Y is the peak area and C denotes the concentration in μg/mL of the tested compound. Characteristic parameters of the linear calibration curve are summarized in Table I.

Table I
Characteristic Parameters for the Regression Equation of the Proposed Method for the Determination of MTD

Parameters	MTD
Calibration range (μg/mL)	0.1–30
Detection limit (μg/mL)	0.027
Quantitation limit (μg/mL)	0.082
Regression equation (Y)[a]: slope (b)	48.8743
Standard error of the slope	0.4382
Intercept (a)	7.3456
Standard error of the intercept	6.2544
Regression coefficient (r^2)	0.9996

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

The limit of detection and the limit of quantification were determined according to the ICH guidelines for validation of analytical procedures (33), and were found to be 0.027 and 0.082 μg/mL, respectively (Table I).

Extraction recovery, precision and accuracy

The proposed mixed-mode chromatographic method using a single protein-coated column gave high extraction recoveries for MTD in the range of 98.61–99.39%. The precision and accuracy

for the proposed method were determined for both intra- and interday variations and expressed as the RSD % and RE % of the mean measured concentration. At three different concentrations, the intraday repeatability and accuracy for the serum samples were excellent, with RSD % being in the range of 0.52–1.15% and with mean RE % ranging from −0.65 to −1.32%. At the same concentration level, the interday reproducibility and accuracy were excellent, with RSD % being in the range of 0.56–1.63% and the mean RE % ranged from −0.97 to −2.04%. The intra- and interday precisions and accuracies at QC concentrations are summarized in Table II. Recoveries for MTD in human serum for the intraday studies were found to be 98.90, 98.68 and 99.35% and for the interday studies were found to be 97.96, 98.04 and 99.03% for QC samples at low (1 μg/mL), medium (10 μg/mL) and high (30 μg/mL) concentration levels, respectively (Table II).

Selectivity

The selectivity was investigated by preparing and analyzing five individual human blank serum samples and samples of drug-free human serum spiked with MTD (10 μg/mL). Each serum sample was tested using the described procedure. Good selectivity for the analyte was obtained as evidenced by the good resolution of its peak. No interference by the endogenous compounds at the retention time of MTD was noted in the matrices studied, when the drug-free human serum samples were compared with the drug-free human serum spiked with MTD (10 μg/mL; Figure 1A and B).

Robustness

To determine the robustness of the proposed method, experimental conditions, namely the flow rate, the pH and the concentration of the acetate buffer solution, were purposely altered and the resolution between MTD and the endogenous components of the serum matrix was evaluated. The results indicate that the slight variations (± 1%) in these parameters did not significantly alter the retention factor or the peak area of the compound under study.

System suitability test

To assess the performance of the proposed method, the system suitability parameters including retention time, resolution, capacity factor, selectivity, number of theoretical plates and tailing factor were investigated during the development and the optimization of the method (Table III). The results indicate that the proposed method permitted adequate resolution of MTD from the endogenous serum components (good resolution and selectivity values) within reasonable run-time. The high value of column efficiency or theoretical plates and the low value of peak asymmetry or tailing factor indicated the suitability and the proper selection of the chromatographic conditions (Table III).

Loading capacity and regeneration conditions of the protein-coated column

The loading capacity of the protein-coated column was examined by injecting an aliquot of 600 μL (equivalent to 30 continuous injections of a 20-μL sample volume) of drug-free human serum using a mobile phase consisting of acetate buffer (0.1 M, pH 2.4), at a flow rate of 1 mL/min and at room temperature (23 ± 1°C). The short protein-coated column (50 × 4.0 mm i.d.) employed in the proposed method allowed the proteins and other macromolecules to be effectively removed in a short time. With up to 150 min elution time of the mobile phase, only two peaks, the frontal large eluted peak (proteins, and other large molecules) and the second small peak of the endogenous serum component were rapidly eluted within the range of 2.24 min after which we did not notice any peak or any disturbance of the baseline. These results show that any compound trapped inside the column did not eluted out during each set of experiment (30 consecutive sample injections) within 150 min elution with the acetate buffer. This implies that MTD (retention time of 3.25) is the last eluting compound when loading 30 continuous injections of a 20-μL sample volume under the proposed chromatographic condition.

To maintain the continuation of getting good results, the protein-coated column was routinely regenerated by flushing at the end of each set of experiment (30 injections) with 50% ethanol in water at a flow rate of 1 mL/min for 5 min followed by re-equilibration with the running mobile phase for further 15 min to elute any strongly retained compound from the protein-coated column. Using high concentration of organic solvent (50% ethanol) resulted in a rapid elution of all retained compounds after each set of experiments (30 injections of a 20-μL sample volume). Thus, the protein-coated column could be used for several sets of experiments without any error that may arise from any possible contaminants.

Also, the extraction recoveries of MTD from human serum samples were checked during each set of experiment (30 injections of 20-μL spiked blank serum with MTD). Since, the small molecule, MTD has been eluted after 3.25 min, it could be concluded that the elution cycle can start 4 min after injecting the former serum sample. The recoveries for MTD were all in the range of 97.96–99.35% with RSD in the range of 0.52–1.63% for low, medium and high levels (Table II). These results demonstrated a good resolution of the method of MTD from serum endogenous components and also clarified that MTD is the last

Table II
Precision and Accuracy Validation of MTD Using the Proposed Method

	Concentration (μg/mL)	Mean recovery[a] (% ± RSD)	Mean RE (%)
Intra-assay[a]	1	98.90 ± 0.83	−1.10
	10	98.68 ± 1.15	−1.32
	30	99.35 ± 0.52	−0.65
Inter-assay[a]	1	97.96 ± 1.63	−2.04
	10	98.04 ± 1.31	−1.96
	30	99.03 ± 0.56	−0.97

[a]Average of five determinations.

Table III
System Suitability Test Parameters of the Proposed Method for the Determination of MTD

System suitability parameters	
Retention time	3.25
Resolution (*R*)	3.37
Capacity factor (*K′*)	6.39
Selectivity factor (*α*)	1.56
Number of theoretical plates (*N*)	1308.03
Tailing factor (*T*)	0.74

eluting compound in the serum analyzed during each set of experiment.

Column lifetime

The immobilization of BSA followed by plasma proteins on the TSK gel ODS-80 TM column was developed with the aim of increasing the column stability, so that large numbers of serum samples could be analyzed without causing rapid column deterioration. The number of injections possible could be estimated from the stability data in terms of the total injected volume that caused a 10% change from the original value of the measured parameters such as column backpressure and peak efficiency. It was found that, the protein-coated analytical column had a limited stability, at least 450 injections of 20 μL serum samples could be made before column backpressure and efficiency started to change. An increase in the injected volumes above this level led to gradual pressure build up at the head of the protein-coated column and ultimately to clogging. Accordingly, a protein-coated column should be routinely changed when excessive backpressure is seen. The number of injections performed, before the deterioration of the column performance, can be significantly increased by filtration of the serum samples prior to injection. Thawed samples often contain clots and solid particles, which on injection will give an immediate increase in the column backpressure owing to restrictions of the inlet filter.

Stability

The stability studies of MTD were carried out to ensure the reliability of the results in relation to handling and storing of the serum 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 h and thawed at room temperature for approximately an hour. Triplicate analyses of the samples at each concentration were quantified. Samples were immediately re-frozen 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 serum samples. Also, MTD in serum samples exhibited no chromatographic changes when stored refrigerated at 5°C for 3 days, and at −20°C for 4 weeks. Serum samples were found to be stable at room temperature upon standing for at least 8 h.

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 500 mg MTD (Aldomet tablet, 250 mg/tablet). Figure 1A and C shows chromatograms of a blank serum sample taken from the volunteer before administrating the drug (Figure 1A), as well as the chromatogram of the clinical serum sample collected after 3 h from orally administering 500 mg MTD (Figure 1C). The analyte peak was well separated and had the same retention time as the peak of standard solution of MTD. Although some peaks from the endogenous serum components were present in the chromatogram, they did not interfere with the peak of interest. The serum concentrations of MTD (μg/mL) in five selected male volunteers, collected after 3 h from administrating a single oral dose of 500 mg MTD, are represented in Table IV.

Table IV
Serum Concentrations of MTD in Five Selected Male Egyptian Volunteers

Volunteer	Serum concentrations of MTD (μg/mL) collected after 3 h from administering a single oral dose of 500 mg MTD					Mean[a] (± SD)
1	1.04	1.17	1.08	1.13	1.16	1.12 ± 0.06
2	1.43	1.47	1.66	1.57	1.54	1.53 ± 0.09
3	2.12	2.22	2.30	2.25	2.33	2.24 ± 0.08
4	2.71	2.77	2.73	2.83	2.87	2.78 ± 0.07
5	3.34	3.45	3.50	3.54	3.49	3.46 ± 0.08

[a]Average of five determinations.

Discussion

In bio-analytical chromatography, the main drawbacks of many common HPLC methods for the therapeutic monitoring of drugs in biological fluids are often time-consuming, labor-intensive and/or rather unselective sample cleanup steps. This is because they typically involve manual pretreatment steps to eliminate the complex sample matrix. To achieve higher selectivity and sample throughput with a simultaneous cost reduction and improvement in the overall analytical quality, the cleanup of the complex biological fluids needs to be optimized and automated. These requirements can be accomplished by integrating the sample cleanup process into the HPLC system. For this purpose, a special mixed-mode liquid chromatography using a single protein-coated ODS-80 TM analytical column was employed for this study. Besides direct injection goal, further advantages of the proposed method are rapid processing of sensitive samples, improved precision and safer handling of infectious samples. The combination of protein-coated analytical column with fluorescence detection allows to set up very efficient HPLC system for highly selective online extraction of MTD in human serum and quantification within 4 min only.

For the better conditions of an eco-friendly analytical protocol, the mobile phase should be either free from or in low levels of organic solvents. Initial efforts to develop an isocratic elution system for the separation of MTD in human serum using buffers only as mobile phases were successful and resulted in a good resolution of the analyte from the serum endogenous matrix components. It was noted that complete separation of MTD and the chromatographic quality were buffer concentration and pH-dependent. Acetate buffer gave the best performance and was selected in all further experiments. Adjustment of the mobile phase pH and buffer concentration was necessary to improve the resolution between the MTD and the endogenous components of the serum matrix.

High extraction recoveries for MTD in the range of 98.61–99.39% were obtained for the QC serum samples. The reason for the quantitative recovery by the proposed method may be

due to the fact that deproteinization was carried out chromatographically, without solvent extraction or without precipitation of serum proteins in contrast to the classical deproteinization procedures (10–12, 14–16, 21). Accordingly, the internal standard could be safely eliminated in the proposed method. This feature is considered to be one of the most important advantages over the classical HPLC methods. In addition, the present method is easy to automate because it allows for a high throughput of samples, without any labor-intensive sample treatment step. Repeatability and reproducibility of MTD in serum samples with high and low concentration levels were below the value of 2.10%, indicating a reliable measurement using the proposed method. These results indicated that the protein-coated analytical column possesses a non-adsorptive outer particle surface when interacting with the macromolecular matrix components (e.g., proteins and nucleic acids). On the other hand, MTD has a free access to the binding centers and thus, can be selectively extracted and separated. The results indicated also that the coated (immobilized) proteins were mainly adsorbed on the outside surface of the porous silica and the inside surface was not coated with proteins owing to the molecular sieve effect of the small pores. The deproteinization in the protein-coated column could be considered as size exclusion due to the small pores of the packing materials. Serum proteins could not be adsorbed further on the external surface of the protein-coated column, and they could not enter the interior of the small pores, thus they flowed out of the column. However, the small pores still retained adsorptivity for small molecules. Accordingly, MTD could be adsorbed on the internal surface of the small pores for a certain period of time, and eluted by the mobile phase to the detector for the final quantification. This combination of size-exclusion chromatography and reversed-phase chromatography of the protein-coated analytical column allows the extraction of the drug directly and selectively from the native biological fluids.

A high degree of confidence in the validity of the proposed HPLC method was showed in the suitability of this technique to monitor the concentration of MTD in serum in five selected male Egyptian volunteers after a single oral dose of 500 mg MTD (Aldomet tablet, 250 mg/tablet), as there were no potential concomitant interferences arising from the matrices. The preliminary experiments showed that the concentration of MTD in volunteers determined by this method agreed with the values obtained from an alternative method (15), making the current technique promising for pharmacokinetic studies in humans.

Conclusion

In this study, an automated HPLC system with a dual-purpose protein-coated analytical column for sample clean up and separation was introduced for the first time to determine MTD in human serum. The combination of mixed-mode chromatography, size-exclusion chromatography and reversed-phase chromatography, using a single protein-coated analytical column, allows the extraction of the drug directly and selectively from the native human serum. The current method has several advantages over other bio-analytical chromatographic techniques since complicated sample pretreatment procedures are avoided along with a minimized human error and thus, a low concentration of MTD can be efficiently separated and determined without the fear of a possible loss during sample preparation protocols. No interference in the assay from any endogenous components was observed. The reduced sample handling and the short runtime made it possible to analyze 15 samples/h. Validity of the method was studied and the method was precise and accurate within a linearity range from 0.1 to 30 μg/mL. The high sensitivity and selectivity of the mixed-mode chromatographic procedure make it a suitable technique for the analysis of MTD in human serum samples. The use of the acetate buffer as the mobile phase and the minimum generation of wastes offered by the proposed method made this work a contribution to the environmental-friendly analytical chemistry. All steps of the conventional sample pretreatment procedures such as extraction and cleanup are integrated into one step and one device, considerably simplifying the sample preparation procedure.

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