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A rapid high performance liquid chromatographic determination of methyldopa in human serum with fluorescence detection and alumina extraction: Application to a bioequivalence study

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Abstract

A simple and ultra rapid high performance liquid chromatographic (HPLC) method coupled with alumina extraction and fluorescence detection was described for determination of methyldopa in human serum. The drug and an internal standard were adsorbed onto alumina and eluted using acidic methanol. The eluate was directly injected onto ODS reverse phase column using a mixture of phosphate buffer (0.05 M) containing triethylamine (100 μl/l, v/v; pH 2.3) and methanol (92:8, v/v) at a flow rate of 2.1 ml/min as the mobile phase. The fluorescence detector excitation and emission wavelengths were set at 270 and 320 nm, respectively. No interference in the assay from any endogenous substances or other concurrently used drugs was observed and the retention times of I.S. and the drug were 1.7 and 2.4 min, respectively with total run time (injection to injection) of less than 3.5 min. The limit of quantification was evaluated to be 20 ng/ml. Validity of the method was studied and the method was precise and accurate with a linearity range from 20 ng/ml to 5000 ng/ml. This method has been used in a randomized crossover bioequivalence study of two different methyldopa preparations in 24 healthy volunteers.

Keywords: Reverse phase chromatography; HPLC; Methyldopa; Bioequivalence study

1. Introduction

Methyldopa (L-α-methyl-3,4-dihydrophenylalanine) 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 stimulation of central α-adrenoreceptors by this agent [1]. Numerous analytical techniques including spectrofluorimetric [2–4] and chromatographic [5–11] methods are now available for analysis of methyldopa in biological fluids. Urinary and plasma concentration of the drug most commonly have been measured by high performance liquid chromatography (HPLC) using electrochemical [7–9,11], mass [5], fluorescence [6] or UV [11] detections. Sample preparation in most of these methods achieved by solid phase extraction [6], protein precipitation [5,7,8] or a tedious, time consuming alumina adsorption [10,11]. Different run time of analysis between the range of 5.5–35 [5,6] min and various quantification limits (20 ng/ml [5], 10 ng/ml [6], 0.8 μg/ml [7], 50 ng/ml [9], 8 μg/ml [10], 10 ng/ml [11]) using different volumes of injection (40 μl [5], 100 μl [6,11], 20 μl [7,9], 1 μl [10]) and samples (200 μl [5], 1 ml [6–9,11], 30 ml [10]) have been reported for analysis of the drug in biological fluids. Analytical conditions in the published methods consisted of gradient [6] or isocratic elution of mobile phase with [6,7] or without using of an ion pair agent. In bioequivalence studies however, the proposed method should be simple and able to process hundreds of samples in a limited time. Furthermore, the assay should be sensitive down to 20 ng/ml. Present paper describes a simple, very rapid and sensitive method for analysis of methyldopa in human serum using alumina extraction. This method in which sample preparation, run time of analysis and LOQ have been improved was successfully used in a bioequivalence study of two different methyldopa preparations.

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2. Experimental

2.1. Reagents and chemicals

Methyldopa and epinephrine (I.S.) were purchased from Sigma (St. Louis, MO, USA). Acid-washed alumina, HPLC-grade methanol, monobasic sodium phosphate, phosphoric acid and triethylamine were obtained from Merck (Darmstadt, Germany). Various medications which have been used to test for interference of the assay were obtained from their respective pharmaceutical manufactures. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Preparation of standards

Stock standard solutions of methyldopa (200 μg/ml) and epinephrine (200 μg/ml) were prepared by dissolving the drugs in methanol and stored in a refrigerator at 4 °C. Methyldopa stock solution was further diluted with methanol to obtain the different working solutions ranging from 0.2 to 50 μg/ml. Working standard solution of epinephrine was prepared by diluting the stock solution with methanol to yield final concentration of 5 μg/ml. Calibration curves were prepared within the concentration range of 20–5000 ng/ml. In disposable glass tubes (16 mm × 100 mm) 100 μl of each working standard methyldopa solutions were evaporated under gentle stream of nitrogen at 50 °C. The residues were reconstituted in the 1 ml human blank serum and following mixing for 10 s on a vortex mixer, the samples were subjected to extraction and analysis.

2.3. Instrumentation

The liquid chromatograph system consisted of two pumps of Shimadzu LC-10A solvent delivery system, a system controller (SCL 10AD), a spectroflurometric detector (RF-551) operated at an excitation wavelength of 270 and emission wavelength of 320 nm, an auto injector (SIL 10A), a column oven (CTO-10A) set at 60 °C, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. The analytical column employed was a reverse phase column (150 mm × 4.6 mm I.D.) which was packed with 5 μm particles of ODS packing material (Shimpack–CLC-ODS) and protected by a Shim-pack G-ODS guard column (10 mm × 4.0 mm I.D., 5 μm particle size). The guard column was routinely changed to avoid excessive pressure build-up in the system. The mobile phase was comprised of methanol–0.05 M phosphate buffer containing triethylamine (100 μl/l) adjusted to pH 2.3 with *o*-phosphoric acid (8:92, v/v). The eluent was filtered through a 0.45 μm filter (Milipore, Bedford, MA, USA) and degassed before use. A flow rate of 2.1 ml/min at a pressure of 155 kg/cm^2 was used.

2.4. Extraction procedure

Serum samples (1 ml, unknown, blank or calibration) were transferred into 1.5 ml propylene centrifuge tubes. To each of these tubes, 100 μl of the I.S. (5 μg/ml) and 50 mg aluminum oxide were added and the resulting mixture was vigorously vortex-mixed (30 s), followed by centrifugation (11,000 × *g*, 3 min). The supernatant fraction was aspirated and discarded and the remaining solid phase was washed with distilled water and subsequently with a 200 μl acidic methanol (HCl 10 *N*-methanol; 1:100, v/v) to desorb the drug and I.S. The acidic eluate was transferred entirely into a 200 μl auto sample vial and a volume of 20 μl was injected into the HPLC system.

2.5. Validation of the method

For method validation and linearity studies, blank serum samples obtained from healthy volunteers were used. To examine the possible interferences of endogenous compounds twenty-four human serum samples from different volunteers were extracted and analyzed during method validation. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. The selectivity of the assay was evaluated by analysis of a group of potentially co-administrated drugs with methyldopa. The efficiency of the extraction procedure of methyldopa at the concentration range of calibration curve as well as the I.S. at the applied concentration were calculated in replicates ($n = 5$) by comparing the respective peak areas of the chromatograms of the extracted samples relative to the untreated standards containing an equivalent amount of the compounds in methanol. Calibration curves (un-weighted regression line) were constructed by linear least-squares regression analysis plotting of peak-area ratios (methyldopa/I.S.) versus the drug concentrations. The limit of detection was defined as the concentration of drug giving a signal-to-noise ratio of 3:1. The limit of quantification was determined as the lowest serum concentration of methyldopa quantified with a coefficient of variation of less than 20%. Intra and inter-day variations were determined by repeated analysis ($n = 6$) of different methyldopa concentrations within the range of calibration curve in a single analytical run and in ten analytical run performed on different days, respectively, using the same stock solutions and plasma batches. Stability of solutions of methyldopa and the I.S. was studied at the applied concentrations over a period of 60 days by comparing of the peak areas at different times. Stability of methyldopa in serum samples was studied by comparing of the determined concentration at different times up to 60 days after storage at −80 °C following three freeze-thaw cycles.

2.6. Application of the method

The present method was used to determine the serum concentrations of methyldopa in a randomized crossover bioequivalence study. Twenty-four healthy volunteers received a single oral dose of 500 mg methyldopa from either Exir (methyldopa; Tehran, Iran) or MSD (Aldomet; UK) pharmaceutical companies in fasting conditions. After 2 week wash-out period the subjects were crossed-over. The blood sampling was carried-out at suitable intervals up to 12 h after drug administration. Pharmacokinetic parameters were calculated and compared using paired Student's *t*-test. Statistical significance was defined at the level of $p < 0.05$.

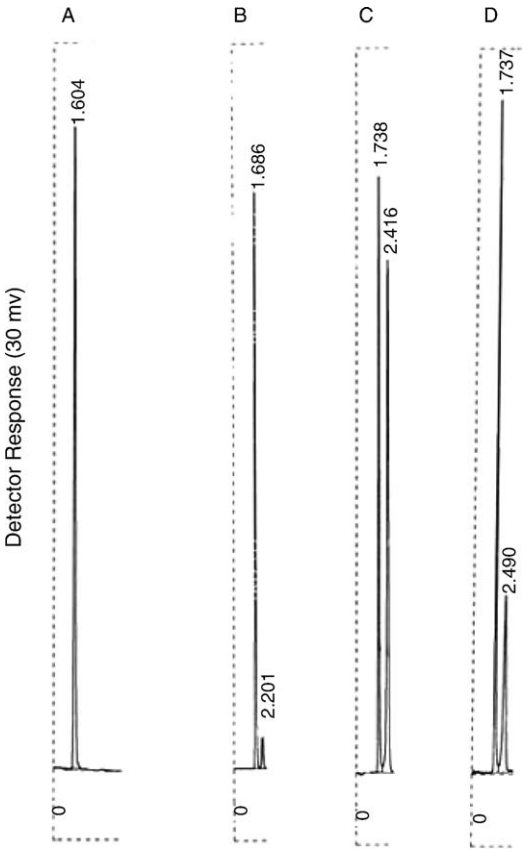

Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum containing the I.S; (B) human blank serum spiked with 40 ng/ml methyldopa and the I.S.; and (C) and (D) serum samples obtained at 3 h and 8 h after a single oral dose of 500 mg methyldopa from a healthy volunteer containing 1280 and 364 ng/ml of methyldopa, respectively.

3. Results

3.1. Specificity and selectivity

The method demonstrated excellent chromatographic specificity with no endogenous serum interference at the retention times of methyldopa and the I.S. Representative chromatograms for human blank serum and human serum spiked with methyldopa (40 ng/ml) and the I.S. are shown in Fig. 1A and B, respectively. Methyldopa and the I.S. were well resolved with good symmetry with respective retention times of 1.7 and 2.4 min and an analytical run time of less than 3.5 min. Fig. 1C and D show the chromatograms of serum sample obtained at 3 and 8 h after a single oral dose of 500 mg methyldopa from a healthy volunteer. The results of the selectivity study showed that the following drugs were not interfered with the described analytical method: levodopa, carbodopa, phenylpropanolamine, phenylephrine, acetaminophen, theophylline, omeprazole, cimetidine, naproxen, indometacin, celecoxib, ibuprofen, diclofenac, clonidine, codeine, caffeine, diltiazem, aspirin, salicylic acid fursemide, amiodarone, cinnarizine, ticlopidine, glybenclamide, verapamil and propranolol.

3.2. Method performance

3.2.1. Recovery, precision, accuracy and stability

The recoveries of methyldopa and the I.S. from serum were determined by extraction of spiked serum samples compared with peak areas obtained after the same amounts of unextracted methyldopa solutions in methanol. The mean recoveries were found to be $98 \pm 3\%$ for methyldopa and $93 \pm 4\%$ for the I.S. The intra-day and inter-day precision and accuracy of the assay were examined by analyzing replicate serum samples spiked with different amounts of the drug within calibration curve range on the same day and on 10 different days. The intra-day and inter-days accuracy and precision values of the assay method are presented in Tables 1 and 2, respectively. The coefficient of variation values of intra day and inter days were less than 12.8% and 13.2%, respectively whereas the accuracy of the method was 97.5–105.5% (intra-day) and 97.1–105.3% (inter-day).

Stock solutions of methyldopa and epinephrine were stable at least for 60 days when stored at 4 °C. Extracted serum was found to be stable for at least 10 h if the samples were kept at room temperature (20–30 °C). The concentrations of methyldopa in serum stored at −80 °C for 60 days and following three freeze-thaw cycles were found to be $100 \pm 2\%$ from the initial values.

3.2.2. Sensitivity and linearity

The detection limit for methyldopa was approximately 8 ng/ml at a signal-to-noise ratio of 3:1 and the quantification limit corresponding with a coefficient of variation of less than 20% was 20 ng/ml using 1 ml serum sample and 20 μl injection. The standard calibration curves were linear over the concentration ranges of 20–5000 ng/ml using line-fit plot in regression analysis with a coefficient of 0.9976 and regression equation of $y = 0.0665x + 2.157$. Intra and inter-day reproducibility for calibration curves were determined on the same day in replicate ($n = 4$) and on different days ($n = 10$), respec-

Table 1
Intra-day precision and accuracy for determination of methyldopa in human serum by the HPLC method

Known concentration (ng/ml)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%mean deviation)
Within-day ($n = 6$)			
20	18.8 ± 2.4	12.8	−5.5
80	78.0 ± 3.5	4.5	−2.5
300	294.2 ± 6.0	2.0	−1.9
1250	1239.2 ± 15.3	1.2	−0.9
2500	2475 ± 24.8	1.0	−1.0
5000	4986 ± 80.4	1.6	−0.3

Table 2
Inter-day precision and accuracy for determination of methyldopa in human serum by the HPLC method

Known concentration (ng/ml)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%mean deviation)
Between-day ($n = 6$)			
20	18.6 ± 2.8	13.2	−5.3
80	78.6 ± 3.9	4.8	−2.9
300	292.1 ± 7.8	2.6	−2.2
1250	1232 ± 14.5	1.4	−1.2
2500	2471 ± 22.6	1.3	−1.4
5000	4988 ± 72.6	1.2	−0.2

tively, using same pooled serum sample. The intra-day average slope of the fitted straight lines was 0.0665 ± 0.0088 ng/ml (C.V. = 10.5%) and the mean intercept of the calibration curves was 2.157 ± 0.6425 (C.V. = 13.5%). The corresponding mean (±S.D.) coefficient of the linear regression analysis was 0.99769 ± 0.0042 (C.V. = 0.65%). For calibration curves prepared on different days, the mean ± S.D. of results were as follows: slope = 0.0725 ± 0.0094 ng/ml (C.V. = 4.3), coefficient of the linear regression analysis = 0.9964 ± 0.030 (C.V. = 0.40%) and intercept = 3.1 ± 0.6232 (C.V. = 10.8%).

4. Discussion

Analysis of methyldopa in biological samples has frequently been reported. Like other catecholamines the drug is not soluble in most of organic solvents thus, sample preparation of methyldopa has been achieved using protein precipitation [5,7,8], solid phase extraction [6] or alumina adsorption [10,11]. In the method described by Oliveira et al. using protein precipitation followed by extraction of lipophilic impurities by dichloromethane and MS detection LOQ of 20 ng/ml and run time of 5.5 min were obtained. Rona et al. obtained LOQ of 10 ng/ml and analytical run time of 35 min using 100 μl injection. The gradient elution of a mobile phase containing an ion pair agent and solid phase extraction were used in their method. Sensitive method with LOQ of 0.8 ng/ml using an ion pair agent in the mobile phase and electrochemical detection has been reported by Lucarelli et al. However in their method total run time of analysis was long (about 12 min) and extraction of the drug has been achieved using protein precipitation followed by evaporation of the aqueous phase. Dilger et al. obtained LOQ of 50 ng/ml using complex extraction method and electrochemical detection. Two tedious multi steps and time consuming methods for extraction of the drug in urine [10] and plasma [11] using alumina adsorption with LOQ of 10 ng/ml with 100 μl injection and 8 μg/ml using 30 ml urine sample and 1 μl injection have been reported. In the present paper unlike previously published complex alumina adsorption method, simple extraction procedure was used and using fluorescence detection obtained LOQ in our method was sufficient for single dose bioequivalence studies.

5. Application of the method and conclusions

This method has been used for analysis of the drug in human serum following single dose administration of 500 mg of two methyldopa preparations in twenty-four healthy volunteers. The mean serum methyldopa concentrations versus time curve of one formulation is shown in Fig. 2. A C_{max} of 1362 ± 804 ng/ml was reached at 2.9 ± 1.1 h after administration, AUC_{0-t} and $AUC_{0-\infty}$ were 5320 ± 2888 and 5479 ± 2921 ngh/ml, respectively, and the drug was eliminated with terminal half life of 1.9 ± 1.0 h.

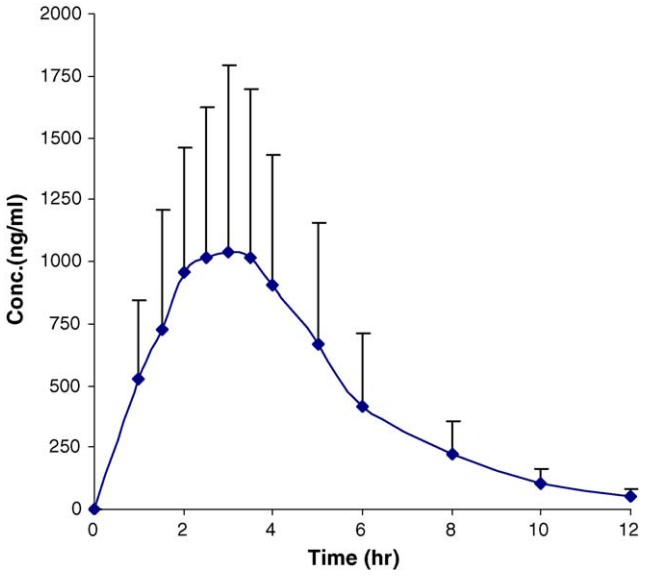

Fig. 2. Mean serum concentrations–time profile of methyldopa in 24 human volunteers after administration of a single oral administration of 500 mg of the drug.

In conclusion a rapid, simple, and sensitive method has been described in the present paper. In this method which has been demonstrated to be suitable for use in pharmacokinetic studies of methyldopa comparing with the previously published method less time is needed for analysis of the drug and simple extraction procedure was used.

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