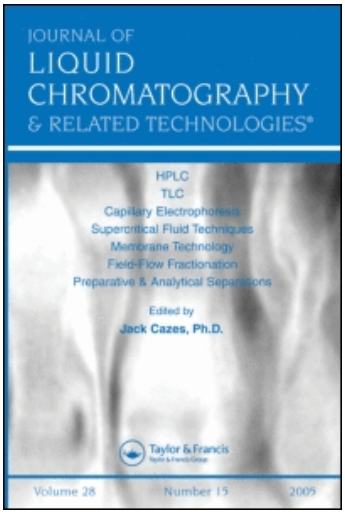



**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Determination of Metyrosine and Its Metabolite in Serum by Reversed Phase High Performance Liquid Chromatography Using Solid Phase Extraction and Fluorescence Detection

M. M. Hefnawy[a]; J. T. Stewart[a]
[a] Department of Medicinal Chemistry, College of Pharmacy The University of Georgia, Athens, GA, USA

**To cite this Article** Hefnawy, M. M. and Stewart, J. T.(1997) 'Determination of Metyrosine and Its Metabolite in Serum by Reversed Phase High Performance Liquid Chromatography Using Solid Phase Extraction and Fluorescence Detection', Journal of Liquid Chromatography & Related Technologies, 20: 18, 3009 — 3016
**To link to this Article: DOI:** 10.1080/10826079708006576
**URL:** http://dx.doi.org/10.1080/10826079708006576

J. LIQ. CHROM. & REL. TECHNOL., 20(18), 3009–3016 (1997)

# DETERMINATION OF METYROSINE AND ITS METABOLITE IN SERUM BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING SOLID PHASE EXTRACTION AND FLUORESCENCE DETECTION

Mohamed M. Hefnawy, James T. Stewart*

Department of Medicinal Chemistry
College of Pharmacy
The University of Georgia
Athens, GA 30602-2352, USA



## ABSTRACT

A novel and rapid method for the separation and determination of metyrosine and its major metabolite alpha-methyldopa in serum by high performance liquid chromatography with fluorescence detection is reported. The methods involved a solid-phase extraction of the two analytes and the internal standard dopamine using a Bond-Elut strong cation-exchange (SCX) column. The eluate obtained from the SCX column is then chromatographed on a reversed phase octadecylsilane column (Spherisorb 0DS2, 250 x 4.6 mm I.D.) with a 92.5:5:2.5 v/v/v 0.1 M aqueous phosphate buffer pH 3-acetonitrile - methanol mobile phase containing EDTA and heptane sulfonate. The flow rate was 1.0 mL/min with excitation at 282 nm and a 370 nm emission filter. The detection and quantitation limits were 1.0 μg/mL and 0.1 μg/mL for metyro-

sine and alpha-methyldopa, respectively, using 1 mL of serum. Linear calibration curves of 5-35 μg/mL and 0.2-2.5 μg/mL for metyrosine and alpha-methyldopa, respectively, show coefficients of determination of more than 0.9995. Precision calculated as %RSD and accuracy calculated as % error were within 2.5 - 6.5% and 2.8-4.2%, respectively, for metyrosine and 4.1-6.3% and 1.3 - 1.5%, respectively, for alpha-methyldopa.

## INTRODUCTION

Metyrosine is an orally active inhibitor of catecholamine synthesis peripherally as well as centrally. It is used to control hypertension in patients with phaeochromocytoma and may be given as a pre-operative preparation to those patients for whom surgery is contra-indicated.[1] Its major serum metabolite alpha-methyldopa has similar activity. [2] A review of the literature revealed that metyrosine is determined in biological fluids and tissues principally by gas chromatography-mass spectrometry.[3] It has also been determined fluorometrically in biological fluids using a liquid-liquid extraction procedure.[4] It has been determined in dosage forms, either polarographically through treatment with nitrous acid [5] or colorimetrically via its reaction with 4-aminoantipyrine in the presence of an alkaline oxidizing agent.[6] The USP 23 recommends a non-aqueous titration method, with potentiometric detection of the end point for the evaluation of the bulk drug material.[7] Thus far, no HPLC methods have been reported for the determination of metyrosine and alpha-methyldopa in serum.



This paper describes a reversed phase HPLC method using fluorescence detection and solid phase extraction (SPE) to measure low μg/mL concentrations of metyrosine and its metabolite in serum, with good sensitivity, selectivity and fast chromatographic run time. The assay procedure possesses the required sensitivity to be useful for monitoring blood levels of metyrosine at 1 gm doses.

## EXPERIMENTAL

### Reagents and Chemicals

Metyrosine (alpha-methyl-L-tyrosine) was obtained from Fluka (Biochemika, AS, USA). Alpha-methyldopa [3-(3,4-dihydroxy phenyl)-2-methyl-L-alanine] was purchased from Sigma (St. Louis, MO, USA). The

internal standard dopamine hydrochloride was obtained from USP (Rockville, MD, USA). Blank bovine serum (Cat # 3160-34) was obtained from Instrumentation Lab (Lexington, MA, USA). Acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA) and were HPLC grade. All chromatographic solutions were filtered through a 0.45 μm filter (Alltech, Deerfield, IL. USA)and degassed prior to use.

**HPLC Conditions**

Chromatography was performed on an isocratic HPLC system consisting of a Beckman Model 110A solvent delivery module (Beckman, San Ramon, CA, USA) and a Spectroflow 980 fluorescence detector (Kratos Analytical, Ramsey, NJ, USA) with excitation at 282 nm and a 370 nm emission filter. Data acquistion was performed on a HP Model 3290 integrator (Hewlett Packard, Avondale, PA, USA). The stationary phase was a 250 X 4.6 mm id Spherisorb ODS2 5μ column (Metachem Technologies, Inc., Torrance CA., USA) at ambient temperature (23°C). The mobile phase was prepared by dissolving 30 mg of 1-heptanesulphonic acid sodium salt-1-hydrate, 10 mg of the disodium salt of EDTA, 1.379 gm of $NaH_2PO_4x$ $H_2O$, 2.5 mL methanol and 5 mL acetonitrile in 100 mL water. The pH of the mobile phase was adjusted to 3.0 with 0.1M phosphoric acid and the flow rate was set at 1.0 mL/min. Quantitation was based on linear regression analysis of analyte peak height versus analyte concentration.

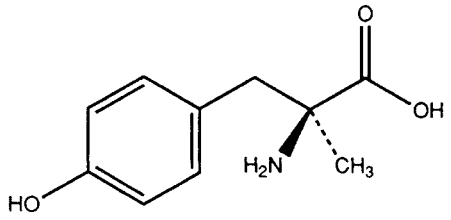


**Preparation of Standard Solutions**

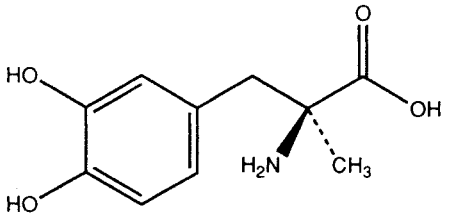
Individual stock solutions of metyrosine, alpha-methyldopa and the internal standard dopamine were prepared in methanol to give concentrations of 100 μg/mL. Appropriate volumes of the two analytes and the internal standard were placed into 1 mL volumetric flasks and drug free serum added to volume to give final serum concentrations of 5, 10, 15, 20, 25, 30, and 35 μg/mL for metyrosine, 0.2,0.5, 1.0, 1.25, 1.5, 2.0, and 2.5 μg/mL for alpha-methyldopa and 2 μg/mL of the dopamine.

**Assay Procedure and Preparation of Standard Curve**

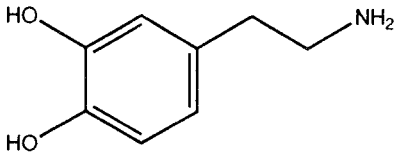
A 1-mL volume of distilled water was added to 1-mL serum samples containing metyrosine, its metabolite and internal standard. For an unknown sample, 20 μL of the internal standard solution was added to 1 mL of serum




Metyrosine (I)




alpha-Methyldopa (II)




Dopamine (III)

**Figure 1**. Chemical structures of metyrosine (I), alpha-methyldopa (II) and dopamine (internal standard, III).

sample followed by the addition of 1-mL of distilled water. The samples were vortex-mixed for 2 min and then passed through a 1-mL SCX Bond-Elut SPE column attached to a vacuum manifold (Vac-Elut, Varian Sample Preparation Products, Harbor City, CA, USA) which was previously conditioned with 2-mL of methanol followed by 2-mL of 0.1 N hydrochloric acid. The column was washed with 2-mL water and allowed to air dry for 3 min. The analytes of interest were eluted with 4 x 250 μL of 1 M diibasic potassium phosphate ( pH adjusted to 5.0 with 1 M phosphoric acid) and a 100-μL aliquot was injected into the HPLC system.

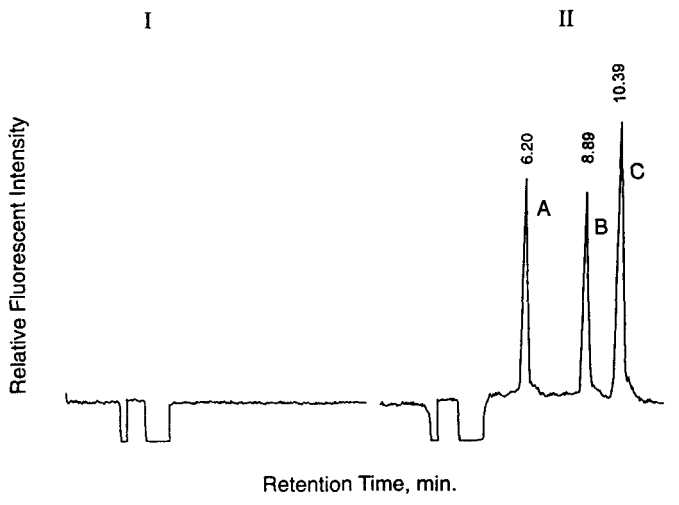



**Figure 2**. Representative chromatograms of (I) serum blank and (II) serum sample with alpha methyldopa (2 μg/mL), (B) dopamine (IS, 2 μg/mL) and (C) metyrosine (30 μg/mL). See Experimental section for HPLC conditions.



Linear calibration curves were constructed in the range of 5-35 μg/mL and 0.2-2.5 μg/mL for metyrosine and alpha-methyldopa, respectively. Linear regression analysis of drug/internal standard peak height ratios versus concentration gave slope and intercept data for each analyte which were used to calculate the concentration of each analyte in the serum samples.

For absolute recovery experiments of each analyte, spiked samples were compared to unextracted stock solutions. Drug peak height ratios were used to calculate the recoveries.

## RESULTS AND DISCUSSION

The chemical structures of metyrosine, alpha-methyldopa, and dopamine (internal standard I.S.) are shown in Figure 1. Metyrosine and alpha-methyldropa show native fluorescence at 370 nm with excitation at 282 nm. Attempts were made to separate metyrosine and alpha-methyldopa on a cyanopropyl column with mobile phases consisting of various portions of acetonitrile-methanol and water. None of these mobile phases were successful in our laboratory due to the lack of stability of the cyanopropyl column. However, an octadecylsilane column enabled the analytes to be separated within

**Table 1**

**Accuracy and Precision Data for Serum Samples with Added Metyrosine (M) and Alpha-Methyldopa**

| Compound | Concn. Add (μg/mL) | Concn. Found (μg/mL)[a,b] | Error (%) | R.S.D. (%) |
|---|---|---|---|---|
| **Intra-day** | | | | |
| M | 8.0 | 7.99 ± 0.530 | 0.13 | 6.63 |
| | 32.0 | 32.59 ± 1.11 | 1.84 | 3.41 |
| A | 0.7 | 0.72 ± 0.047 | 2.85 | 6.35 |
| | 2.2 | 2.15 ± 0.056 | 2.27 | 2.61 |
| **Inter-day** | | | | |
| M | 8.0 | 8.23 ± 0.544 | 2.87 | 6.57 |
| | 32.0 | 30.65 ± 0.770 | 4.22 | 2.51 |
| A | 0.07 | 0.68 ± 0.043 | 1.57 | 6.32 |
| | 2.2 | 2.17 ± 0.090 | 1.36 | 4.14 |

[a] Based on n=3 for intra-day assay.
[b] Based on n=9 for inter-day assay.



a reasonable chromatographic run time at a 1 mL/min flow rate. The final mobile phase composition for the analysis of metyrosine and alpha-methyldopa was 92.5:5:2.5 v/v/v 0.1 M aqueous phosphate buffer pH 3-acetonitrile-methanol containing EDTA and heptanesulfonate. Figure 2 shows representative chromatograms of a serum blank and serum spiked with the two analytes and dopamine internal standard.

An SPE procedure was developed for sample cleanup to decrease the sample preparation time normally seen in liquid-liquid extraction. Octadecylsilane and octylsilane SPE columns were initially investigated and they gave very low recoveries of the two analytes. There were several advantages to the use of a strong cation-exchange (SCX) column for serum cleanup. In addition to an almost interference-free chromatographic analysis, the extraction procedure can be adapted for batch processing by using a Vac-

Elut chamber, which allowed the processing of ten serum samples simultaneously in less than 15 min. Under the SPE process described in this paper, the SCX column can withstand two column volumes of water washes, so that the non-polar components are washed off the column without affecting the recoveries of metyrosine and alpha-methyldopa.

The calibration curves showed good linearity in the ranges 5-35 μg/mL and 0.2-2.5 μg/mL for metyrosine and alpha-methyldopa, respectively. The coefficients of determination were more than 0.9995 for metyrosine and alpha-methyldopa, respectively. Representative linear regression equations obtained for metyrosine and alpha-methyldopa were $y = 0.0546x + 0.02821$ and $y = 0.78683x + 0.00027$, respectively, where y and x are the D/IS peak-height ratios and concentration of each analyte, respectively. The intra-day precision (n=3) as expressed by percent RSD and percent error (accuracy) was 3.41-6.63% and 0.13 -1.84% for metyrosine and 2.61-6.53% and 2.27-2.85% for alpha-methyldopa. The inter-day precision and accuracy (n=9, over three days) were 2.51-6.57% and 2.87-4.22% for metyrosine and 4.14-6.32% and 1.36-1.57% for alpha-methyldopa, respectively. The detailed data is listed in Table 1.



In summary, a precise, accurate and rapid HPLC method using isocratic conditions and employing solid-phase extraction and fluorescence detection has been developed for the analysis of metyrosine and its major metabolite alpha-methyldopa. The method is sensitive to 1 μg/mL and 0.1 μg/mL for metyrosine and alpha-methyldopa, respectively (S/N = 2). The total chromatographic run time of the isocratic method was less than 11 min.

## ACKNOWLEDGMENT

The authors thank the Egyptian Cultural and Educational Bureau, Washington, DC for financial assistance.

## REFERENCES

1. R. N. Brogden, R. C. Heel, T. M. Speight, A. S. Avery, Drugs, **21**, 81-89 (1981).

2. A. G. Gilman, T. W. Rall, A. S. Nies, P. Tayler, **The Pharmacological Basis of Therapeutics**, 8th Ed. Pergamon Press, Inc., (1990) p 789-793.

3. B. Sjoequist, Biomed. Mass Spectrom., **6**, 392-398 (1979).

4. F. A. Aly, F. Belal, A. El-Brashy, Pharm. World Sci., **15**, 206-211 (1993).

5. F. A. Aly, M. I. Walash, F. Belal, Anal. Lett., **27 (4)**, 2677-2687 (1994).

6. M. M. Hefnawy, F. A. Aly, F. Belal, Anal. Lett., **28 (10)**, 1811-1818 (1995).

7. **The United States Pharmacopeia 23 National Formulary 18**, The United States Pharmacopeial Convention, Rockville, MD 1995, p. 1022.

Received March 1, 1997
Accepted April 1, 1997
Manuscript 4392