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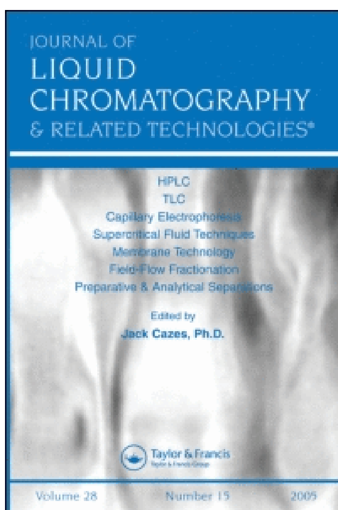

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Morad, A. M. , Al-meshal, I. A. , El-feraly, F. S. and Matar, Kamal M.(1988) 'High Performance Liquid Chromatographic Determination of (-)-N-Formylnorephedrine in Plasma', Journal of Liquid Chromatography & Related Technologies, 11: 3, 713 — 724

To link to this Article: DOI: 10.1080/01483918808068339

URL: http://dx.doi.org/10.1080/01483918808068339

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JOURNAL OF LIQUID CHROMATOGRAPHY, 11(3), 713-724 (1988)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF (-)-N-FORMYLNOREPHEDRINE IN PLASMA

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ABSTRACT

An HPLC procedure for the detection and quantitative estimation of (-)-N-formylnorephedrine in rabbit plasma had been developed. The procedure involved the extraction of (-)-N-formylnorephedrine from plasma spiked with the internal standard (phenacetin), using ethyl acetate. The ethyl acetate extract is evaporated under nitrogen and the residue is reconstituted in water and injected onto the column. A u-Bondapak-C18 column 30 cm x 3.9 mm ID was used. The mobile phase is 20% acetonitrile in water; at a flow rate of 1.5 ml/min and uv detection at 256 nm. A linear relationship between concentration and peak height ratio (I/internal standard) was obtained (r = 1.00). The reported procedure allows the measurement of (-)-formylnorephedrine in concentrations as low as 150 ng/ml of plasma with total procedure time of about 10 min. The applicability of the procedure to pharmacokinetic studies is illustrated and metabolites are shown not to interfere with the assay procedure.

INTRODUCTION

Khat (Cathaedulis Forsk) is a shrubby plant grown in some countries of Central Africa and in the southern parts of the Arabian peninsula.

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The fresh leaves of khat are chewed by some local inhabitants for their stimulant amphetamine-like effects. The increased cultivation and consumption of khat resulted in serious socio-economic problems in these areas. The latter, have stimulated interest in the isolation, identification, structure illucidation and pharmacological studies on khat leaves constituents (1,2,3,4).

In studies carried out in our laboratories; the results of which were reported earlier (5), a new compound; (-)-N-Formylnorephedrine (I) was isolated from khat leaves. Its structure was illucidated (Fig. 1) and its synthesis was also undertaken. This compound is presently under investigation for its pharmacologic and toxicological effects in laboratory animals. It has an amphetamine-like structure and preliminary pharmacological screening showed that it has similar stimulant effect in laboratory animals. Also, estimation of its pharmacokinetic parameters in laboratory animals is being investigated.

The purpose of the present report is to describe a high performance liquid chromatographic technique (HPLC) for the quantitative determination of I in aqueous solutions and in rabbit plasma.

MATERIALS AND METHODS

Apparatus

A waters HPLC Unit (Waters Associates, Milford, MA) was used. It consisted of an M-45 model pump; an auto-injector (Wisp-710B); system controller model M-720 UV, detector Model M-480 and a data module model M-730. Stainless steel, u-Bondapak C_{18} column 30 cm x 3.9 mm I.D. (Waters Associates, Milford, MA.)

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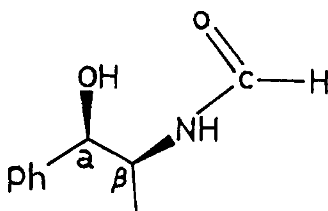

Fig. 1 : (-)-N-Formylnorephedrine.

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Chemicals and Reagents

All chemicals and reagents were of analytical grade. Acetonitrile is of HPLC grade and ethylacetate is spectro-grade.

Chromatographic Conditions

A mobile phase containing 20% v/v acetonitrile in water was used. The flow rate was 1.5 ml/min and the detector was set at a wavelength of 256 nm and 0.002 (AUFS) for sensitivity. Experiments were conducted at ambient temperature.

Stock Solutions

A stock solution of I in methanol was prepared at a concentration of 2 mg/ml. A stock solution containing 2 mg/ml phenacetin (internal standard) in methanol was prepared. This solution was diluted to a concentration of 10 ug/ml with water before use.

Preparation of Aqueous Standard Solutions

The stock solution of compound I containing 2 mg/ml in methanol was diluted with water to obtain a working solution containing 100 ug/ml. This solution was appropriately diluted with water to obtain standard

solutions containing 1,2,4,6, and 8 ug/ml of I. To each 500 ul of these solutions, 8 ul of the internal standard solution was added.

Assay Solutions

60 ul of the aqueous solution; containing I and to which 8 ul of 10 ug/ml phenacetin was added is injected onto the column.

Preparation of Standard Solutions in Rabbit Plasma

Rabbit plasma, obtained from freshly drawn blood, was spiked with the appropriate amounts of both I and the internal standard (prepared from the stock solutions described above). In this way standard solutions were obtained containing 1,2,4,6 and 8 ug/ml of I and 0.16 ug/ml of the internal standard. These solutions were extracted as described below, and an appropriate volume of the extract was injected onto the column.

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Extraction of I From Rabbit Plasma

To 500 ul of rabbit plasma containing 0.16 ug/ml of the internal standard, 5 ml of ethyl acetate was added, vortex for 1 min. then centrifuge for 5 min at 4000 rpm. A 4 ml aliquot of the organic layer is transferred to a test tube and the solvent is evaporated to dryness at $60^{o}C$ under a stream of nitrogen. The residue was reconstituted into 350 ul of water and an appropriate volume of this aqueous solution, usually 60 ul, was injected onto the column.

Efficiency of Extraction

A known quantity of compound I was added to a suitable volume of freshly separated rabbit plasma to yield concentrations of 1,2,6 and 8 ug/ml. 500 ul of each of these standards was then extracted as des-

cribed above and 60 ul of the extract was injected onto the column. The peak height ratios obtained were then compared to those obtained from similar aqueous solutions, omitting the extraction step.

RESULTS AND DISCUSSION

The chromatogram of (-)-N-formylnorephedrine (I) and the internal standard in aqueous solution is shown in Fig. 2 (A). Compound I showed peak (a) with a retention time of 4.3 min, the internal standard (phenacetin) showed a peak (b) and a retention time of 9.6 minutes. Also shown the chromatogram (B) following the injection of rabbit plasma extract obtained from fresh rabbit plasma spiked with both I and the internal standard; peaks (a) and (b) are for compound I and the internal standard, respectively, with the same retention times as that observed with the aqueous solutions.

The pharmacokinetic studies on compound I involved its administration to rabbits by various routes, namely; rapid IV injection, IV infusion and orally. In an animal experiment, involving the oral administration of I in a dose of 60 mg/kg to a 3.8 kg rabbit, blood samples were drawn at time internals over a period of two hours following drug administration. Fig. 3 shows the chromatograms obtained from a plasma extract of a blood sample taken 60 min (A) and 75 min (B) following drug administration. Peaks (a) and (c) are for compound I and the internal standard (retention times: 4.3 and 9.6 min) respectively. The appearance of peak (b) at a retention time of 7.8 min is attributed to a possible metabolite of compound I. Comparison of the two chromatograms, (A) and (B) in Fig. 3, for the two samples, obtained from the same rabbit at 60 and 75 min post drug administration, shows the reduction of peak

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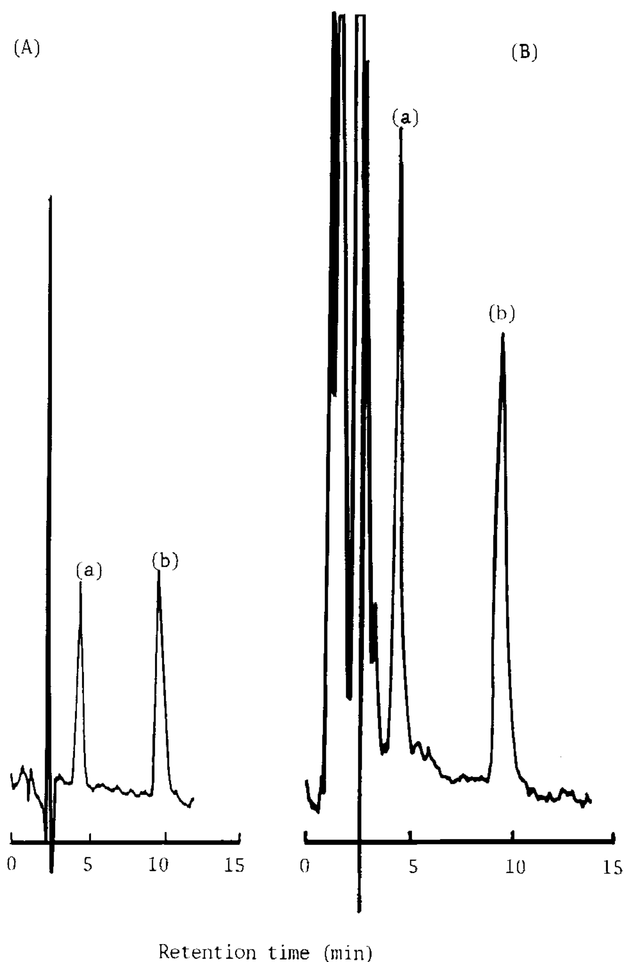

Fig. 2 : Chromatograms of I in aqueous solution (A) and in rabbits' plasma extract (B). Peaks (a) and (b) are for compound I and the internal standards respectively. Chromatographic conditions; Column -u Bondapak-C_{18}, 30 Cm x 3.9 mm ID; mobile phase - 20% acetonitrile in water; flow rate - 1.5 ml/min; detection - UV 256 nm; injection volume 30 ul of aqueous solution, 60 ul of rabbit plasma extract; internal standard-phenacetin 0.16 ug/ml; sensitivity - .002 AUFS.

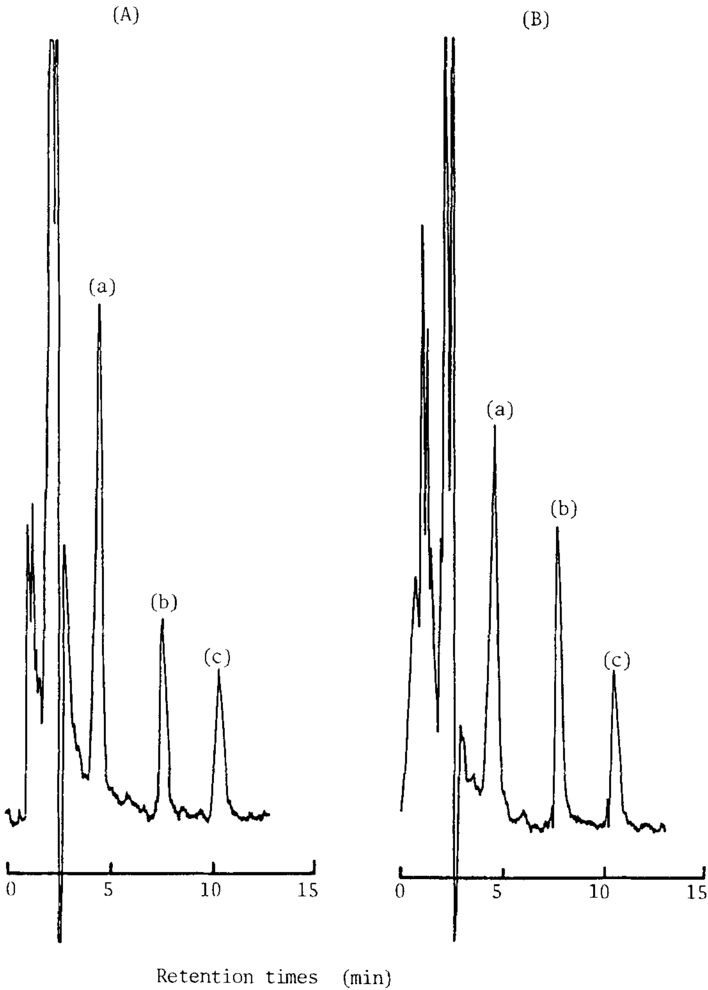

Fig. 3 : Typical chromatogram of rabbit plasma extract following the oral administration of 60 mg/kg I to a 3.8 kg rabbit. Blood samples were drawn 60 mins (A) and 75 mins (B) after administration of I. Peaks : (a) compound I, (b) is a metabolite and (c) is internal standard. Chromatographic conditions are as under Fig. 2 and in text.

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height ratio (I/internal standard) i.e., peak, (a), from 3.2 to 2.5. Also an increase in peak height ratio (metabolite/internal standard) i.e., peak (b), from 1.3 to 1.9. This progressive increase in peak height ratio of the metabolite (b), was consistently observed in all animals used in pharmacokinetics studies.

A plot of peak height ratio (I/internal standard) for standard solutions of I in water is shown in Fig. 4. A straight line relationship is obtained. Linear regression analysis of experimental data points showed a linear relationship (Y = 0.12 x) and an excellent correlation coefficient (r = 1.00). A plot of peak height ratio (I/internal standard) for standard solutions of I in rabbit plasma is shown in Fig. 5. A straight line relationship is obtained (Y = 0.17 x) and an excellent correlation coefficient (r = 1.00). It is also observed that concentrations of 1 ug/ml and less could be accurately measured.

The efficiency of the extraction procedure is shown in Table 1 below.

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Table 1

Efficiency of Extraction of I From Rabbit Plasma :

Concentration of standard I (ug/ml).	Peak height ratio (I/internal standard) in water.	Peak height ratio (I/internal standard) from rabbit plasma extract.	Percent recovery.
1	0.17	0.175	102.9
2	0.34	0.35	102.9
6	0.9	1.00	111.1
8	1.19	1.35	113.4
Mean ± SD.			107.6 ± 5.5

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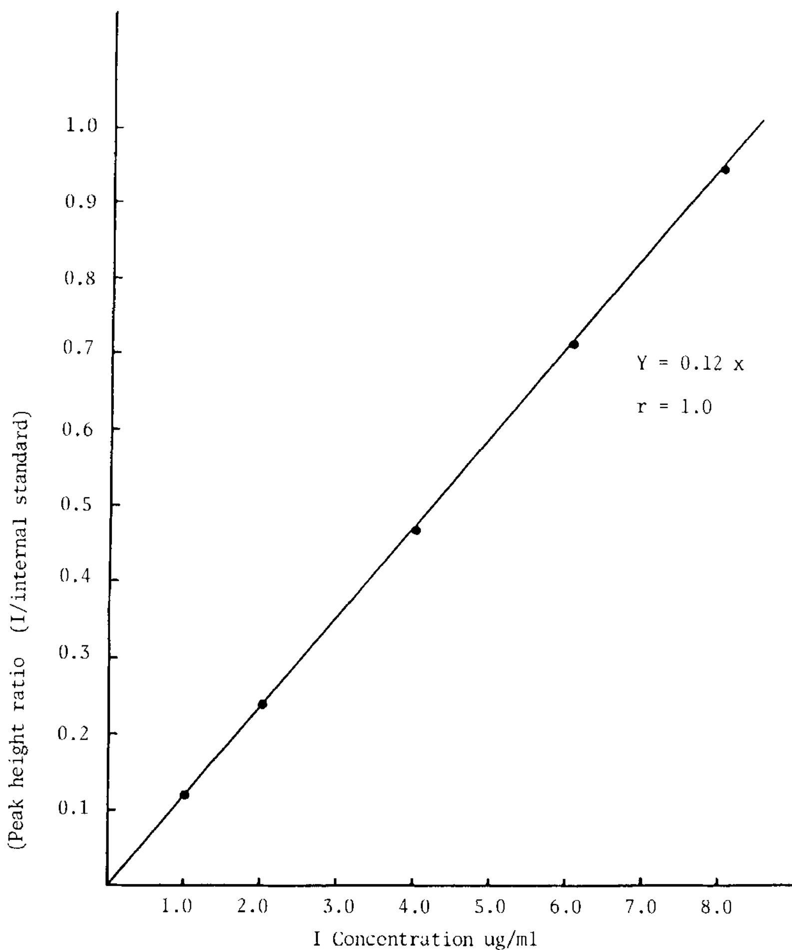

Fig. 4 : Plot of peak height ratio vs I concentration in aqueous solutions. Chromatographic conditions are as under Fig. 2 and in text. (Y = 0.12 x, r = 1.0).

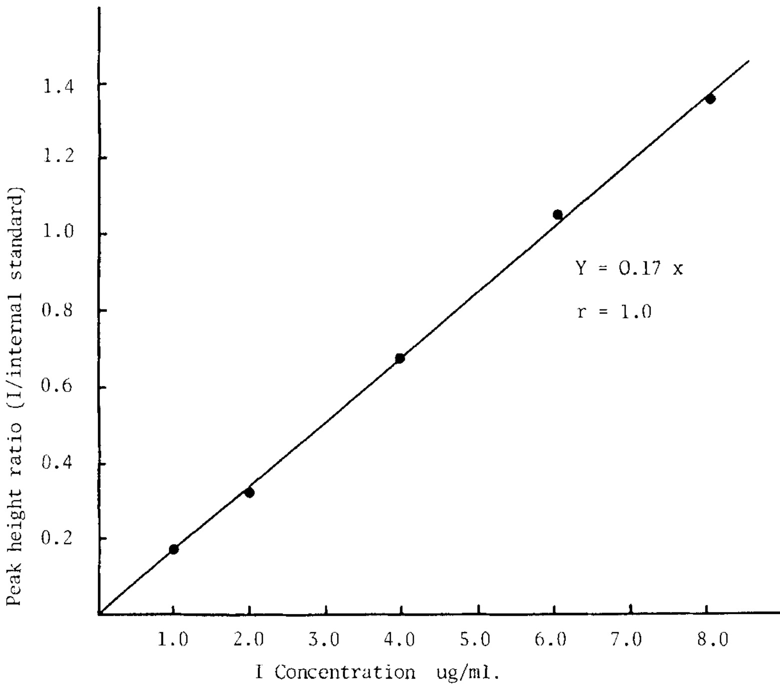

Fig. 5 : Plot of peak height ratio vs. I concentration in rabbits plasma. Chromatographic conditions are as mentioned under Fig. 2 and in text. (Y = 0.17 x, r = 1).

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The applicability of the assay procedure to pharmacokinetic studies is illustrated in Fig. 6. A dose of 60 mg/kg of I was injected into a 3.8 kg Newzealand rabbit, and blood samples taken at various time intervals, were analysed for compound I. An excellent fit of data points to a two compartment open model is shown for the pharmacokinetic behaviour of this compound thus illustrating the utility of the outlined assay procedure in pharmacokinetic studies involving (-)-N-formylnorephedrine.

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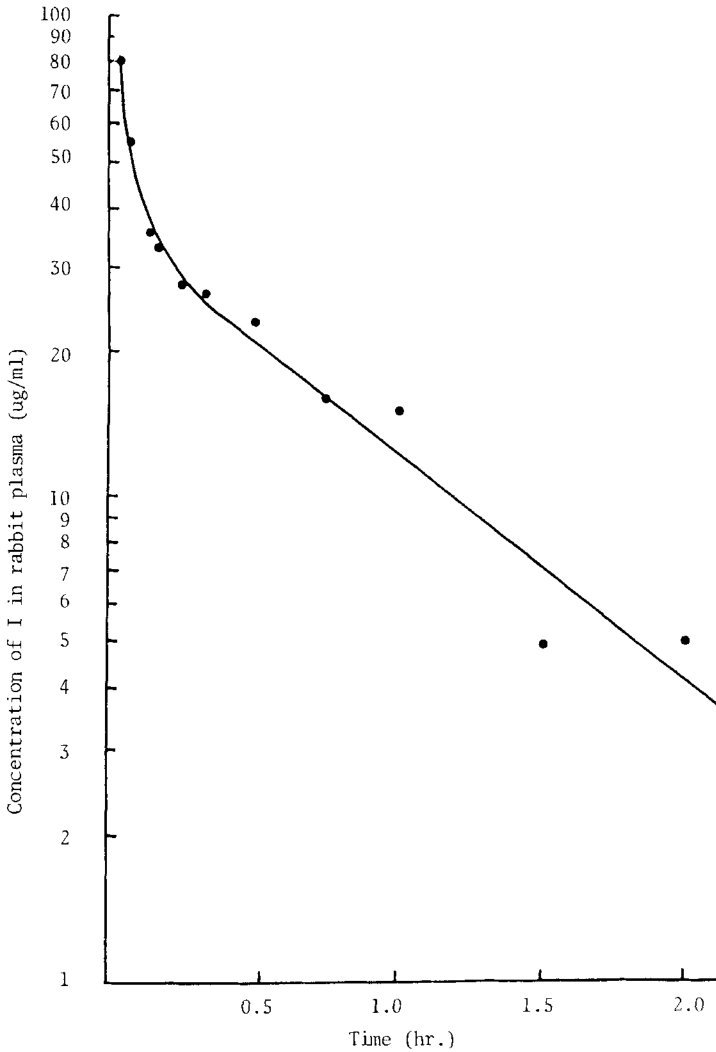

Fig. 6 : Blood level vs time following the rapid intravenous injection of 30 mg/kg of I into rabbit. Solid line is concentration-time profile predicted by the two compartment open model and solid circles are experimental data points.

CONCLUSION

A sensitive and selective method for the detection and quantitative estimation of (-)-N-formylnorephedrine had been developed. Levels as low as 150 ng/ml could be quantitated in plasma, without interference from possible metabolites, which makes the study of the pharmacokinetics of this new compound possible.

ACKNOWLEDGEMENT

This Project was supported by a grant number DSE-1982-1 from King Abdulaziz City for Science and Technology (KACST).

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