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Short communication

Quantification of 2,3-dihydro-7-methoxypyrrolo-[2,1-b]-quinazolin-9(1H)-one, a new antiallergic agent, by high-performance liquid chromatography in serum☆

Shio Kumar Singh, Jyoti Kumar Paliwal, Pyara Krishen Grover, Ram Chandra Gupta*

Pharmacokinetics and Metabolism Division, Central Drug Research Institute, Lucknow 226 001, India

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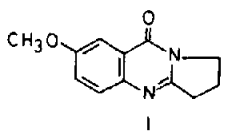
Abstract

The CDRI compound 73/602 (I, 7-methoxydeoxyvasicinone) is an antiallergic agent presently undergoing Phase II clinical trials. A rapid, precise and sensitive high-performance liquid chromatographic assay has been developed and validated for the detection of I in rat serum. The method involves extraction of rat serum samples with diethyl ether after addition of 1 *M* potassium hydroxide. Separations were accomplished on a RP-18 column using acetonitrile-phosphate buffer (pH 7) (20:80, v/v) as the mobile phase. Recovery of I was always >90%. An excellent linear relationship ($r = 0.9999$) was obtained between the quantified and added concentrations over the range of 2.5 to 500 ng/ml. The sensitivity of the assay for 0.5 ml serum was 2.5 ng/ml.

1. Introduction

2,3-Dihydro-7-methoxypyrrolo-[2,1-b]-quinazolin-9(1H)-one (I, Fig. 1) is a new antiallergic agent under development in our Institute. It is a structural analogue of vasicinone, previously extracted from the leaves and roots of *Adhatoda vasica* (Acanthaceae) [1] and has been widely used in the Indian system of medicine as an expectorant and mild bronchial antispasmodic [2–4]. Compound I, like sodium chromoglycate shows no antagonistic activity against acetylcholine, serotonin and histamine [5, 6].



I

Fig. 1. Chemical structure of CDRI compound 73/602 (I).

Currently I is undergoing Phase II clinical trials as an antiallergic agent. In anticipation of the analysis of serum samples for toxicologic and pharmacokinetic studies, a selective and sensitive high-performance liquid chromatographic (HPLC) method was developed for the quantitation of I in serum. The assay was used for quantitation in rat serum after oral administration.

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* Corresponding author.

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2. Experimental

2.1. Reagents and standards

Compound I was obtained from the Pharmaceutics Division of the Central Drug Research Institute. Its purity was determined to be 99.99%. The working standard solutions were prepared by diluting a stock solution of I (100 μg/ml in acetonitrile) with mobile phase. Dipotassium hydrogen phosphate (Sarabhai Chemicals, Baroda, India), orthophosphoric acid (Glaxo, Bombay, India), and potassium hydroxide (S.D. Fine Chemicals, Bombay, India) were of analytical grade and used without further purification. HPLC grade acetonitrile was obtained from S.D. Fine Chemicals (Bombay, India). All glassware was washed with detergent, rinsed thoroughly with triple-distilled water and then dried prior to use.

2.2. Chromatographic conditions

The HPLC instrument consisted of a Model 600 pump (Kontron Electro Lab., London, UK), a Model 7125 injector with a 100-μl loop (Rheodyne, Berkeley, CA, USA) and a Model RF-530 variable-wavelength fluorescence detector (Shimadzu, Kyoto, Japan). Separation was accomplished on a 100 × 4.6 mm I.D., 5-μm C_{18} cartridge analytical column preceded by a 30 × 4.6 mm I.D. guard column (Pierce Chemical, Rockford, IL, USA). The column eluent was monitored at an excitation wavelength of 275 nm and an emission wavelength of 363 nm. Chromatograms were recorded and integrated by a Model CR1B Chromatopac integrator-plotter (Shimadzu, Japan). A model SVC-200H Speed-Vac concentrator (Savant Instruments, New York, NY, USA) was used to evaporate the organic solvent after extraction.

The mobile phase consisted of acetonitrile–50 m*M* dipotassium hydrogen phosphate (adjusted to pH 7 with orthophosphoric acid) (20:80, v/v). It was filtered and degassed before use. Chromatography was performed at ambient temperature at a flow-rate of 1 ml/min. The retention time of I was 6.6 ± 0.2 min.

2.3. Stock and standard solution preparation

Stock solution containing 100 μg/ml was prepared in acetonitrile and stored at 4°C. The appropriate dilutions of the stock solution in mobile phase were analysed at regular intervals over a period of 6 months. There was no significant decrease in the peak height. The working standards were prepared from stock solution in mobile phase in the range 2.5–500 ng/ml.

2.4. Calibration samples

Appropriate quantities of stock solution were diluted with methanol to give solutions in the range of 2.5 to 20 μg/ml. These solutions were used as serum spiking standards. Suitable quantities (10–250 μl) of serum spiking standards were added to 10 ml of drug-free serum. Thus serum concentration ranging from 2.5 to 500 ng/ml were obtained. The standards were vortex-mixed briefly, stored at −20°C and thawed before use.

2.5. Extraction

To 0.5-ml serum samples containing I in a 10-ml (75 mm × 5 mm) glass test tube were added 25 μl of 1 *M* potassium hydroxide. The constituents were extracted twice with 3 ml of diethyl ether. During each extraction, tubes were vortex-mixed for 1 min followed by centrifugation at 1000 *g* for 15 min at 4°C. The ether layer was separated into a conical glass centrifuge tube (110 mm × 14 mm) after snap freezing in liquid nitrogen. The combined ether extracts were evaporated to dryness using the Savant sample concentrator. The residue was reconstituted in 250 μl of mobile phase and analysed by HPLC using a 100-μl injection loop.

2.6. Accuracy and precision

Aliquots (0.5 ml) of serum ($n = 5$), containing 2.5, 10, 50, and 250 ng/ml of I were processed as described. The concentrations were determined against the standard calibration curve in serum. The accuracy of the method was calculated based

on the difference between the mean calculated and added concentrations, while precision was evaluated by calculating the inter-day coefficients of variation (C.V.).

3. Results and discussion

3.1. Chromatography

A decrease in the percentage of acetonitrile in the mobile phase on the RP-18 column caused an increase in the retention time of the compound and reduced the peak sharpness, while the pH of the phosphate buffer system had no significant effect on the peak sharpness. A mobile phase of acetonitrile–50 m*M* dipotassium hydrogen phosphate (pH 7) (20:80, v/v) was found to be optimal for the proper resolution of I from serum endogeneous compounds.

3.2. Selectivity and specificity

Fig. 2 shows chromatograms of control standard containing 100 ng/ml of I in mobile phase (A), an extract of drug-free serum (B), a serum standard containing 100 ng/ml (C), and serum sample from a rat treated with a single 50 mg/kg oral dose of I (D). The retention time of I was 6.6 ± 0.2 min. Serum components eluting prior to the compound I did not interfere with the analysis. The quantitation limit of I in serum was 2.5 ng/ml (C.V. < 10%) after 2 fold concentration using 0.5 ml of serum. This method provided adequate sensitivity and specificity for monitoring serum levels of I.

3.3. Extraction efficiency

Addition of 25 μl of 1 *M* potassium hydroxide was essential to maintain the drug in an unionised form for better extraction efficiency and to avoid the extraction of interfering matrix components. A simple, double extraction with diethyl ether was sufficient to give greater than 90% absolute recovery of the drug (Table 1). To determine the extraction efficiency, a standard solution containing I was added to aliquots (0.5 ml) of serum giving 2.5, 10, 50 and 250 ng/ml of I. The recovery of I was calculated by comparing the peak heights of these extracted samples, reconstituted in 250 μl of mobile phase, with those obtained from the analysis of equivalent amounts of the standard solution in buffer injected directly. A two-fold concentration was necessary to maximise the sensitivity of the assay.

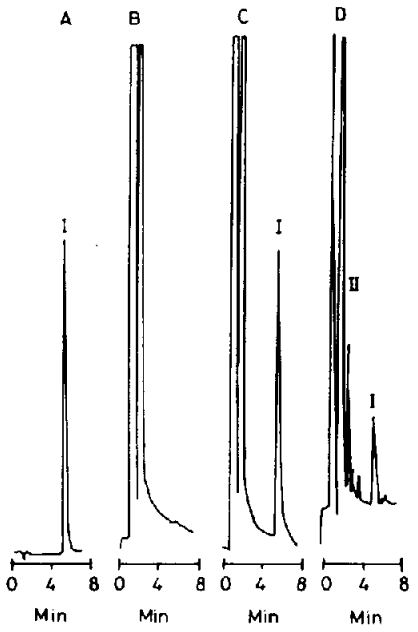

Fig. 2. Chromatograms (A) standard containing 100 ng/ml of I, (B) drug-free serum, (C) serum containing 100 ng/ml of I, and (D) rat serum after 4 h of 50 mg/kg oral dose of I

Table 1
Extraction efficiency of I from spiked serum samples ($n = 5$)

Concentration (ng/ml)	Extraction efficiency (%)	Coefficient of variation[a] (%)
2.5	95.08	6.80
10	97.78	5.92
50	103.24	2.79
250	98.14	1.76

[a] Coefficient of variation = (S.D./mean) · 100.

3.4. Linearity and reproducibility

Linear least square regression analysis of the calibration graph demonstrated linearity in the range 2.5–500 ng/ml. A typical standard curve could be described by $y = 88.32x + 148.5$ ($r = 0.9999$). The reproducibility and accuracy of the method were determined by processing spiked serum samples at 2.5, 10, 50 and 250 ng/ml with respect to the calibration curve run each day. Five samples were analysed for each concentration. The within-day coefficients of variation ($n = 5$) were less than 5% (Table 2). The day-to-day coefficients of variation ($n = 5$) of samples analysed on five different days were less than 7% (Table 2).

3.5. Application of method in clinical pharmacokinetics

The assay method described here was applied to determine the concentration–time profile of I in normal healthy rats after a single oral dose. It is of interest to note that the chromatogram (Fig. 2D) of the serum sample from dosed rats contained an additional major peak at 3.35 min compared to blank rat serum. This peak may be due to a metabolite (Fig. 2D, II) of I. Chromatograms of blank and spiked rat serum are shown in Fig. 2B and 2C. No interfering peaks were present at the elution region of I or II.

References

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Table 2
Precision and accuracy for I

Spiked concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Accuracy (% of mean deviation)
Within-day (n = 5)			
2.5	2.39 ± 0.02	0.75	−4.40
10	10.40 ± 0.23	2.18	+4.00
50	46.11 ± 1.40	3.02	−7.78
250	228.11 ± 3.11	1.36	−8.76
Day-to-day (n = 5)			
2.5	2.38 ± 0.03	1.15	−4.80
10	10.54 ± 0.15	1.50	+5.40
50	49.32 ± 3.0	6.04	−1.36
250	253.36 ± 2.36	0.93	+1.34