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Note

High-performance liquid chromatographic assay for RA 642, a compound with cardiovascular effect, in human and rabbit serum[a]

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2,2′-{[4,8-Bis(diethylamino)pyrimido[5,4-*d*]pyrimidine-2,6-diyl]bis[(2-methoxyethyl)imino]}bisethanol (RA 642, I, Fig. 1) is a compound that has been shown to have cardiovascular and hypertensive effects [1]. Results from studies done in cats have demonstrated the potential applications of I in the management of endotoxic shock [2]. This report describes a high-performance liquid chromatographic (HPLC) analytical method for measuring I in both human and rabbit serum that is suitable to support pharmacokinetic and pharmacodynamic studies.

EXPERIMENTAL

Chemicals and reagents

Compound I was provided by Dr. Walter Kobinger (Ernst-Boehringer Institute for Medical Research, Vienna, Austria) and the dipyridamole (II), used

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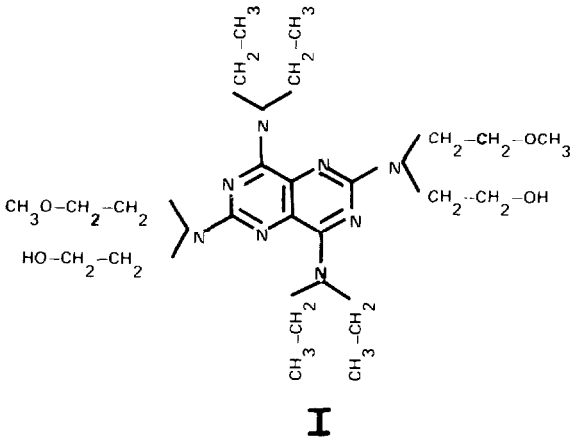
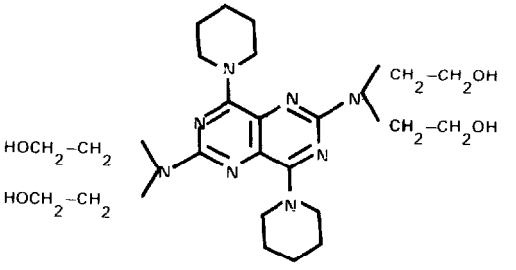


I



II

Fig. 1. Chemical structures of RA 642 (I) and dipyridamole (II).

as the internal standard, was supplied by the manufacturer (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, U.S.A.). HPLC-grade acetonitrile and methanol and reagent-grade sodium phosphate monobasic and sodium phosphate dibasic were used (Mallinckrodt, Paris, KY, U.S.A.). Water used for the analysis was passed first through a reverse osmosis filtration system (Milli-Q Water Purification® system, Millipore, Bedford, MA, U.S.A.) and then through a membrane filtration system (Organicpure® system, Barnstead, Boston, MA, U.S.A.). Human serum was purchased from commercial sources (Irving Scientific, Santa Ana, CA, U.S.A.).

Chromatography

The chromatographic system consisted of a high-performance liquid chromatograph (Hewlett-Packard Model 1082) equipped with a UV photodiode array detector (HP Model 1040A) that operated at 296 nm, an autosampler (HP Model 79842A), and an analytical workstation (HP Model 79994A). The analytical column used was a Spheri-10 reversed-phase C_8, 10 μm particle size, 100 mm × 4.6 mm I.D. column (Brownlee Labs., Santa Clara, CA, U.S.A.) that had a C_8, 10-μm pre-column attached (OS-GU, Brownlee Labs.); both were operated at room temperature.

The mobile phase was run at a flow-rate of 1.5 ml/min and consisted of a 0.01 *M* sodium phosphate buffer (pH ca. 7.0)–acetonitrile gradient system. The acetonitrile concentration increased from 30 to 55% over 5 min, then remained isocratic at 55% for 7 min.

Assay procedure

Stock solutions of I (100 μg/ml) and II (2 mg/ml) were prepared in methanol–water (50:50, v/v) and standards were spiked with these solutions. Spiked human serum standards were prepared containing 0, 3, 5, 10, and 20 μg of I and 10 μg of II (internal standard) in a total volume of 1 ml. These standards were passed through 3-ml disposable octadecylsilane columns (J.T. Baker, Phillipsburg, NJ, U.S.A.). The columns were rinsed twice with 1 ml of water and were then eluted with 1 ml of methanol. The methanol solution was collected in 3-ml tubes and evaporated to dryness under a stream of nitrogen in a water bath (35–40°C). The residue was dissolved in 200 μl methanol and 50-μl aliquots were injected onto the column.

Blood was harvested from New Zealand white rabbits weighing 3–5 kg. The blood was allowed to clot at room temperature and centrifuged at approximately 1000 *g* for 10 min, then the serum was removed. Using this serum, spiked standards were prepared as previously described. Blood samples from rabbits injected intravenously with I were collected from intra-arterial catheters placed in an ear artery, and the serum was separated and prepared in the same manner.

Sample concentrations of I were calculated from linear regression curves of the area ratios of I/II versus concentration of the spiked standards. Recovery of I was calculated by comparing the areas obtained from extracted standards to that of methanolic solutions injected directly onto the column and averaged 80–85%.

RESULTS AND DISCUSSION

Fig. 2 shows the UV spectrum of I in methanol–water (30:70, v/v). Both 230-nm and 296-nm wavelengths gave absorption maxima, but there was less interference from other plasma constituents at the 296-nm wavelength. There-

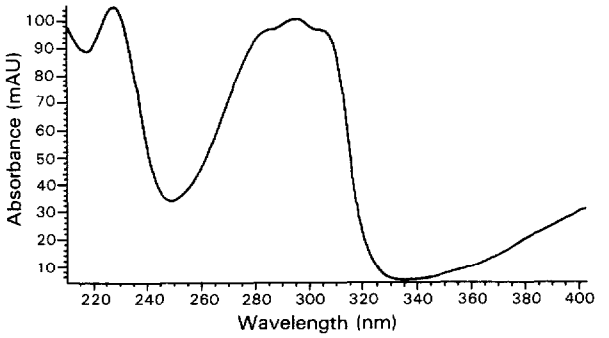


Fig. 2. UV spectrum of I in methanol–water (30:70, v/v) (ca. 10 μg/ml).

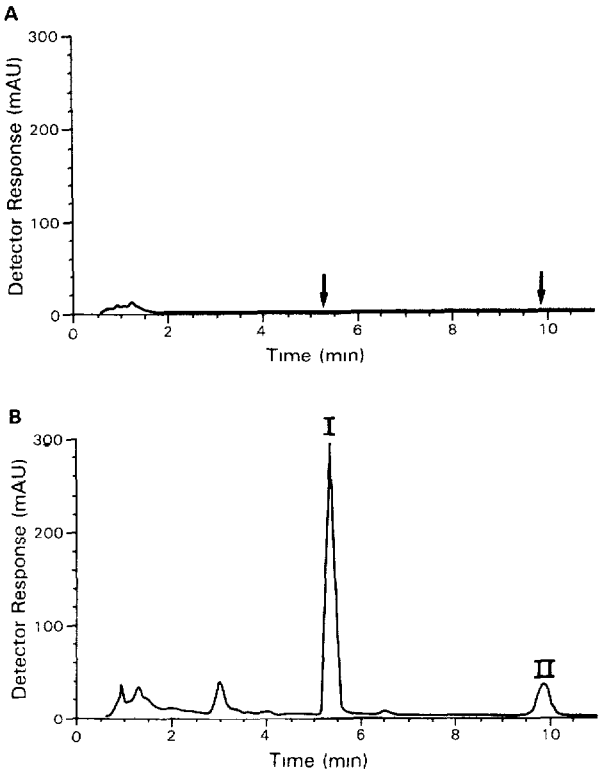


Fig. 3. Chromatogram of human serum blank (A) and human serum standard (B) containing 5 μg of I and 10 μg of II.

fore, quantitation of I in serum was based on the absorption at 296 nm. In addition, this wavelength was suitable for II because of the close structural relationship.

Chromatography

Fig. 3 shows a chromatogram from a human serum blank (Fig. 3A) and from a human serum standard containing 5 μg of I and 10 μg of II (Fig. 3B). Fig. 4A is a chromatogram of a blank rabbit serum and Fig. 4B is a chromatogram of a serum sample (obtained from a rabbit given 4 mg/kg I) containing 4.0 μg of I to which 10 μg of II has been added. The retention times were 5.3 and 9.8 min for I and II, respectively. The small peak occurring at approximately 3 min is due to an impurity present in the internal standard. However, it was necessary to utilize gradient elution chromatography in order to insure that no interfering peaks were seen in either human or rabbit serum.

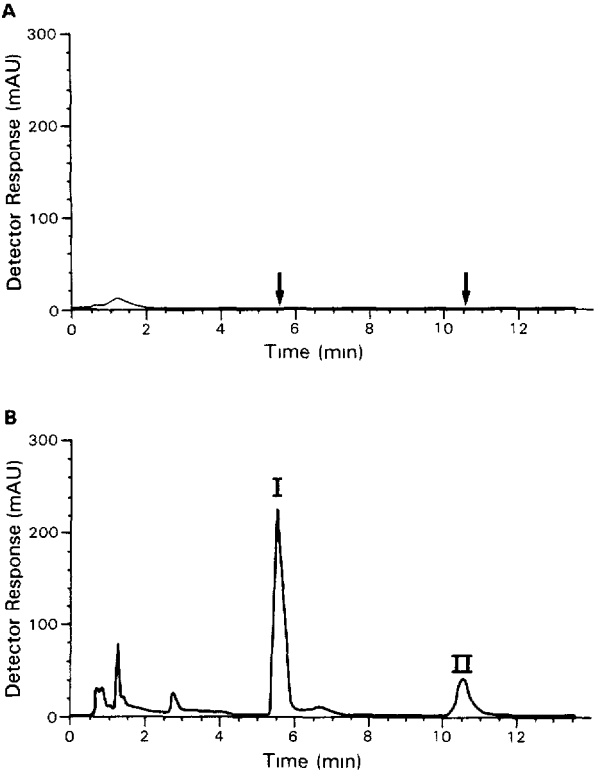


Fig. 4. Chromatogram of rabbit serum blank (A) and rabbit serum standard (B) containing 4 μg/ml I from rabbits given 4 mg/kg I by intravenous injection (10 μg of II added as internal standard).

TABLE I

BETWEEN-DAY ASSAY PRECISION AND ACCURACY FOR I IN HUMAN SERUM

Human serum standards containing I and II were prepared fresh daily from stock methanolic solutions. Values are for ten samples.

Concentration (μg/ml)		Accuracy[a] (%)	Coefficient of variation (%)
Spiked	Measured		
3	2.75	91.53	5.23
5	4.65	93.01	4.25
10	9.70	97.00	4.23
20	20.24	101.20	1.35

[a]Accuracy = 100 × (measured concentration − spiked concentration)/spiked concentration.

Assay linearity and precision

Standard curves prepared from data obtained from either human or rabbit serum were linear over the concentration range 0–20 μg/ml. Least-squares regression analysis of the area ratios of I/II versus concentration of I in human serum over this concentration range was performed on data obtained on ten separate days. The results yielded a slope of 0.0785 ± 0.0014, an intercept of -0.0300 ± 0.0066 (mean ± standard error of the mean), and a correlation coefficient of 0.9983 ± 0.0007. The usable lower limit of sensitivity for this procedure was 3 μg/ml. Essentially identical values were obtained with spiked rabbit serum standard curves. Consequently, for humane reasons, sample quantitation was based on human serum standard curves. Table I shows the between-day reproducibility of spiked human standards.

In summary, we have developed a sensitive and accurate HPLC assay that can measure I in human and rabbit serum and is suitable to support pharmacokinetic and pharmacodynamic studies.

ACKNOWLEDGEMENTS

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REFERENCES

1 R. Kadatz, W. Kobinger and A. Walland, Arch. Pharmacol., 292 (1976) 97.
2 W. Kobinger, A. Walland and R. Kadatz, Arch. Pharmacol., 292 (1976) 105.