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Note

Simple method for the determination of rhein in biological fluids by high-performance liquid chromatography

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Diacerhein (4,5-diacetoxy-9,10-dihydro-9,10-dioxy-2-antracencarboxylic acid) is a new antiarthrosic drug with different characteristics from other drugs used for the treatment of arthrosis. Diacerhein does not inhibit prostaglandins and shows, at molecular and cellular levels, a new mechanism of action [1–7]. Many clinical trials confirm the effectiveness of diacerhein in the treatment of arthrosis (gonarthrosis, coxarthrosis) and degenerative joint disease [8–14]. Diacerhein is completely metabolized by animals and humans into rhein (4,5-dioxy-9,10-dihydro-9,10-dioxy-2-antracencarboxylic acid), which is the active metabolite [4].

A few methods have been described for the quantitative determination of rhein in biological fluids. A fluorimetric method suffers from a lack of sensitivity and specificity [15]; another fluorimetric method is time-consuming and complex [16]. A high-performance liquid chromatographic (HPLC) method has been described for quantitative determination of rhein and its conjugates in urine [17].

This paper describes a simple HPLC method for the determination of rhein in plasma and urine and some results after oral administration of diacerhein to rats.

EXPERIMENTAL

Reagents and materials

The solvents used were all of HPLC grade (LiChrosolv, Merck, Darmstadt, F.R.G. or C. Erba, Milan, Italy). The water was previously bidistilled using a glass distiller and filtered on a 0.45-μm membrane (type FH, Millipore). Rhein standard was prepared in our laboratories; the other reagents were all of analytical grade.

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High-performance liquid chromatography

The apparatus used was a Pye-Unicam LC-3 model, equipped with a UV-50 UV–Vis detector, CDP-4 printer–plotter computer integrator and Model 7125 Rheodyne valve. The column (250 mm × 4 mm I.D.) was packed with LiChrosorb RP-8, 10 μm (reversed phase) and equipped with a PVDF guard column (4 mm × 4 mm I.D.) packed with LiChrosorb RP-18, 5 μm. The temperature of the column was 18°C. The mobile phase was McIlvaine buffer pH 2.2–acetonitrile (55:45, v/v). The pH 2.2 buffer was prepared by mixing 19.60 ml of 0.1 *M* citric acid with 0.40 ml of 0.2 *M* disodium phosphate. The flow-rate was 1.0 ml/min, the amount injected 20 μl and the detection wavelength 258 nm.

Standard solution

A standard solution of rhein was prepared at a concentration of 100 μg/ml in N,N-dimethylacetamide and stored at 4°C. After evaporation of solvent containing rhein (0.25–10 μg), the residue was dissolved in mobile phase (1 ml) and a 20-μl aliquot of the resulting solution was injected into the chromatograph. The calibration curve was obtained by adding known amounts of rhein to rat plasma or urine. These standards were treated as described below.

Assay procedure

To 1 ml of plasma or urine was added 1 ml of acetonitrile. The mixture was stirred on a Vortex mixer for 1 min and centrifuged at 1600 *g* for 5 min and 20 μl of the supernatant were injected into the chromatograph.

Quantitative evaluation

The rhein content in plasma (or urine) was determined by comparison of the sample peak area with peak areas of the calibration curve. No internal standard was used owing to the very simple procedure.

Animal study

Male Wistar rats (Nossan), weighing 200 g and fasted overnight, were used. The animals were orally treated with 25 mg/kg diacerhein suspended in 10% acacia gum solution (10 ml/kg). Blood samples were drawn before and 0.5, 1, 2, 4 and 6 h after the administration. Urine was collected for 24 h. Rhein was assayed as described above.

RESULTS AND DISCUSSION

Figs. 1 and 2 show typical chromatograms of rhein and diacerhein in plasma and urine under our experimental conditions. The retention times of diacerhein and rhein are 7.73 and 9.60 min, respectively. The blank chromatograms show that no interference occurred from endogenous substances in the plasma or urine.

Rhein in plasma and urine was efficiently extracted by acetonitrile. In order to check the validity of the proposed method a known amount of rhein was added to rat plasma and urine, and its recovery rate was determined by the HPLC method. As listed in Table I, the recovery rate of the rhein in plasma was

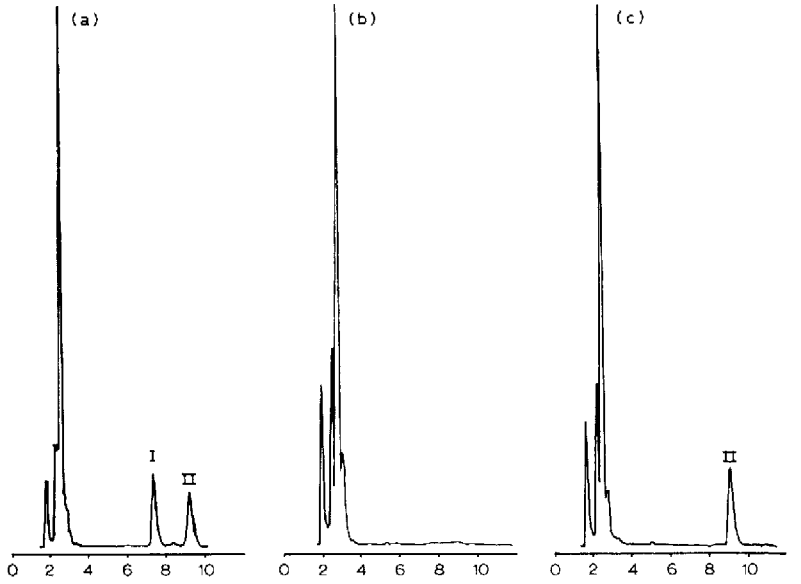

Fig. 1. High-performance liquid chromatograms obtained from (a) plasma spiked with diacerhein (I) (10 μg/ml) and rhein (II) (10 μg/ml), (b) drug-free plasma and (c) plasma of rats treated with *diacerhein. Chromatographic conditions are as described in Experimental.*

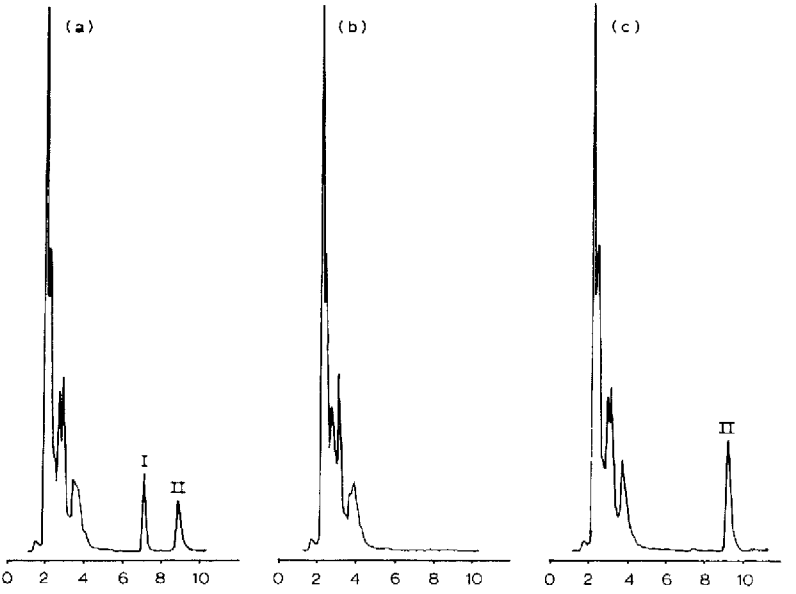

Fig. 2. High-performance liquid chromatograms obtained from (a) urine spiked with diacerhein (I) (10 μg/ml) and rhein (II) (10 μg/ml), (b) from drug-free urine and (c) urine of rats treated with diacerhein. Chromatographic conditions are as described in Experimental.

TABLE I

RECOVERY TEST FOR RHEIN ADDED TO HUMAN PLASMA AND URINE

Sample	Concentration (μg/ml)		Recovery (mean ± S.D., $n=6$) (%)	Difference of mean from theoretical (%)
	Added	Found		
Plasma	0 50	0.489	97.77 ± 5.27	−2.23
	5.00	4.913	98.26 ± 4.71	−1.74
Urine	0.50	0.495	98.93 ± 4.61	−1.07
	5.00	5.014	100.28 ± 4.88	+0.72

TABLE II

DAY-TO-DAY AND WITHIN-DAY REPRODUCIBILITY

Concentration of rhein in plasma (μg/ml)	Coefficient of variation (%)	
	Day-to-day ($n=10$)	Within-day ($n=10$)
0.5	3.40	4.50
2.5	3.05	2.65
5.0	2.70	3.20

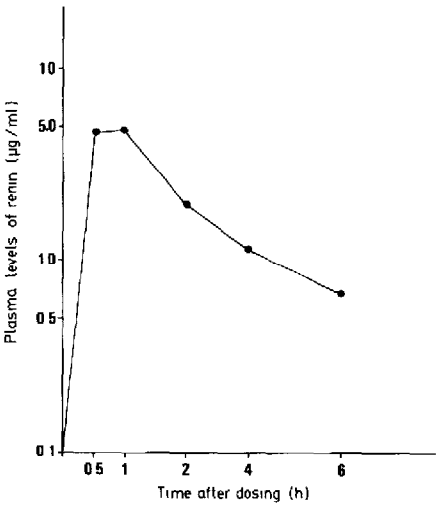

Fig. 3. Plasma levels of rhein in rats after oral administration of diacerhein (25 mg/kg) (means of three rats).

97.77–98.26% and in urine 98.93–100.28%. The accuracy, defined as the difference between the found and true values, is within 2.5% for plasma and 1.5% for urine (Table I).

The calibration curve is linear over the range 0.10–20 μg/ml, both in plasma and in urine. The relationship between rhein urine concentrations in the range 0.1–20 μg/ml and the peak areas is expressed as $y = 24.308x + 635.757$, where x is the amount of rhein injected and y is the peak area. The correlation coefficient is $r = 0.99978$. The detection limit of rhein was estimated as 0.1 μg/ml, with a signal-to-noise ratio of ca. 8:1.

The within-day and the day-to-day reproducibilities of the method were checked at plasma rhein concentrations of 0.5, 2.5 and 5.0 μg/ml. Ten determinations of each were done on the same day. The day-to-day reproducibility was assessed over a period of ten days. The results given in Table II show values within 4.50%.

The first results of bioavailability studies on animals are reported in Fig. 3. The diacerhein is totally metabolized into rhein; the peak plasma level is at 1 h, and the levels are well detectable until 6 h. Diacerhein is not found at any time. All the values are the mean of three rats. The urinary excretion of free rhein from 0 to 24 h after oral administration of diacerhein (25 mg/kg) is $4.90 \pm 0.36\%$.

In conclusion, the method we propose here is simple and sensitive, with a high degree of precision, and can be applied either in drug monitoring or in human and animal pharmacokinetic trials.

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