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Note

Determination of rhein and its conjugates in urine by high-performance liquid chromatography

KATSUYOSHI UCHINO*, YOSHIKAZU YAMAMURA, YUKIYA SAITOH and FUJIO NAKAGAWA

Hospital Pharmacy, Faculty of Medicine, University of Tokyo, 3-1, Hongo 7-chome, Bunkyo-Ku, Tokyo 113 (Japan)

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Recently, the number of patients with hypokalemia and/or pseudo-Bartter's syndrome, caused by abuse of diuretics and laxatives to attain a slim figure, has increased in our country [1—5], making it necessary to analyse the abused drug in the body fluids of patients. Previously, we developed simultaneous qualitative analyses of eleven diuretics using high-performance liquid chromatographic (HPLC) and thin-layer chromatographic (TLC) methods [4], and analyses of three 1,8-dihydroxyanthraquinone derivatives (1,8-OHADs) using HPLC and 1,8-OHAD—aluminium chelation methods [6]. The usefulness of our analyses of diuretics has been evaluated to differentiate between Bartter's syndrome and pseudo-Bartter's syndrome [7].

In laxatives, sennoside A- and B-containing laxative (SL) is a much-abused drug. The drug is metabolized and excreted in the urine as rhein (RH), glucuronide and sulphate of RH in rat and man [8, 9]. However, a few methods have been reported for quantitatively determining RH and its metabolites in biological fluids. An earlier fluorimetric method suffered from a lack of sensitivity and specificity [10]. Another fluorimetric method, involving TLC separation with two solvent systems, was time-consuming and complex [11].

The present paper describes a HPLC method, which has a high sensitivity, selectivity and precision, for the quantitative determination of RH and its conjugates in urine.

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EXPERIMENTAL

Drugs and reagents

Dosage forms of SL were Pursennid® tablets (12 mg; Sandoz, Switzerland), powdered senna, powdered rhubarb (JP X grade, Suzu Pharmaceutical, Osaka, Japan) and Alosenn® granule (Ernst Schworer, F.R.G.). Authentic RH was kindly supplied by Sandoz. 1,8-Dichloroanthraquinone, used as an internal standard (I.S.), was obtained from Wako (Osaka, Japan) and recrystallized from chloroform. β-Glucuronidase (100 U/ml) and arylsulphatase (25 U/ml) were purchased from Boehringer Mannheim (F.R.G.). All other solvents and reagents used were of reagent grade.

Extraction of RH from urine

To 3 ml of urine, 100 μl of a 20 μg/ml solution of I.S. in ethanol were added. After a short time, 3 ml of 0.1 *M* acetate buffer (pH 5.0) and 15 ml of methylene chloride were added to the solution. The mixture was shaken for 10 min and centrifuged at 1680 *g* for 5 min. The organic phase was transferred to another centrifuge tube and evaporated to dryness with a rotary evaporator at room temperature. The extracted residue was dissolved with 100 μl of ethanol and 20-μl aliquots of this solution were injected into the HPLC column.

Conditions of the HPLC method

The HPLC apparatus consisted of a Shimadzu LC-6A liquid chromatograph and a Shimadzu SPD-1 spectrophotometer (Shimadzu, Kyoto, Japan). The UV wavelength for detection of RH was set at 235 nm. The column was a stainless-steel tube (250 × 4 mm I.D.) packed with Senshu gel 7 C_{18} H (Senshukagaku, Tokyo, Japan), coated with ca. 20% C_{18} hydrocarbon, using a column-packing apparatus (Senshukagaku). The column temperature was maintained at 30°C by a column jacket connected to a water bath. The mobile phase was 2% acetic acid—methanol (HPLC grade, Wako) (30:70). About 5 m of PTFE tube connecting the HPLC apparatus was placed in a water bath at 30°C in order to secure a more constant temperature for the mobile phase; it was degassed by sonication before use. The flow-rate of mobile phase was set at 1.0 ml/min (pressure ca. 115 kg/cm^2).

Calibration curves of RH

To prepare a stock solution of RH, 10 mg of authentic RH were dissolved in chloroform in a 500-ml volumetric flask. The solution was diluted with ethanol to produce a standard solution of the desired concentration (0, 100, 250, 500, 1000 or 2000 ng/ml). A 1-ml aliquot of each standard solution of RH was transferred to a 50-ml centrifugal tube, and was added to 3 ml of drug-free urine. The samples were analysed by the method described above. The calibration curve for determination of RH was obtained by plotting the peak-height ratios (RH/I.S.) against the concentration of RH in each spiked sample.

Hydrolyses of RH conjugates

Urine samples were successively hydrolysed by β-glucuronidase, aryl-sulphatase and hydrochloric acid. To remove RH in the urine before each

hydrolysis procedure, the urine was washed into 0.1 *M* acetate buffer (pH 5.0) by a 2.5-fold volume of methylene chloride. The incubation conditions for each hydrolysis were as follows: β-glucuronidase: added volume, 25 μl per 3 ml of urine, time and temperature, 24 h and 37°C; arylsulphatase: added volume, 25 μl per 3 ml of urine, time and temperature, 72 h and 37°C; hydrochloric acid: added volume, 1.2 ml per 3 ml of urine, time and temperature, 2 h and in a boiling water bath. RH in the sample after each hydrolysis was analysed after extraction by the method described above.

Sample collections

One of the authors (male, 38 years, 73 kg) was orally administered, successively, one Pursennid tablet, 1 g of powdered senna, powdered rhubarb and Alosenn granule at 9:00 a.m. every two weeks. He was shown by physical and laboratory examinations to be in good physical condition. Urine after the administration of each drug was collected for 24 h. Its volume was measured and a portion was kept at −20°C until analysis.

Urine from a female hypokalemic patient (56 years, 53 kg) with SL abuse was collected in an outpatient clinic and kept at −20°C until analysis.

RESULTS AND DISCUSSION

Since the extraction and HPLC procedures for the qualitative analysis of three 1,8-OHADs from urine in our previous paper [6] did not have the most desirable conditions for quantitative determination of RH, a procedure for RH was newly developed in the present paper. The use of methylene chloride as an extraction solvent for RH, rather than diethyl ether or ethyl acetate, reduced the amount of endogenous substance in the urine.

Typical chromatograms of RH are shown in Fig. 1. Each peak was sharp and well resolved. The blank chromatogram showed that no interference occurred with endogenous substance in the urine.

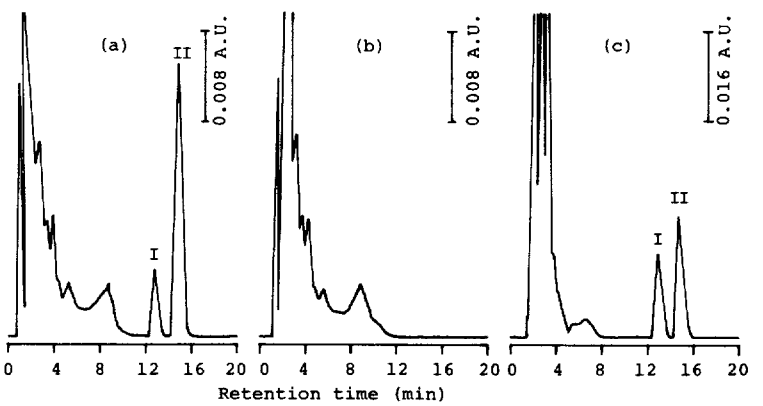

Fig. 1. High-performance liquid chromatograms obtained from 3 ml of urine spiked with 250 ng of rhein and 2 μg of 1,8-dichloroanthraquinone as an internal standard (I.S.) (a), from 3 ml of drug-free urine (b), and from 1 ml of urine of a patient with laxative abuse (c). Peaks: I = rhein; II = 1,8-dichloroanthraquinone (I.S.). Chromatographic conditions are as described in Experimental.

TABLE I

REPRODUCIBILITY OF ANALYSIS OF RHEIN (RH) IN URINE

Added amount of RH (ng)	Peak-height ratio (n = 5)	
	Mean ± S.D.	C.V. (%)
100	0.105 ± 0.004	3.8
250	0.253 ± 0.012	4.7
500	0.517 ± 0.017	3.3
1000	1.000 ± 0.027	2.7
2000	1.992 ± 0.028	1.4

The extraction recovery of RH from urine was determined at three different concentrations. The data at 0.25, 1 and 2 μg of RH per 3 ml were 83.5 ± 0.8%, 82.9 ± 1.1% and 80.8 ± 1.1% (n = 3, mean ± S.D.), respectively. There were no significant differences between values ($P < 0.05$). The precision of analysis for RH was acceptable over the entire range of concentrations. The coefficient of variation (C.V.) of RH ranged from a low of 1.4% at 2000 ng per 3 ml up to 4.7% at 250 ng per 3 ml (Table I).

The calibration curve for RH was linear in the range 100—2000 ng, using 3 ml of urine, and passed through the origin. The lower limit of RH detection in urine was 100 ng per 3 ml with a signal-to-noise ratio of ca. 10:1.

The present method was checked for interference by other drugs possibly abused together with SL. Such drugs were benzylhydrochlorothiazide, bumetanide, chlorthalidone, cyclothiazide, cyclopenthiazide, furosemide, hydrochlorothiazide, hydroflumethiazide, methyclothiazide, trichlormethiazide, polythiazide, emodin and danthron. These drugs were completely removed by extraction and chromatographic separation.

The optimal conditions for each hydrolysis of RH conjugates were determined from preliminary study. The time courses of hydrolysis of RH conjugates in urine after the administration of 1 g of powdered senna by

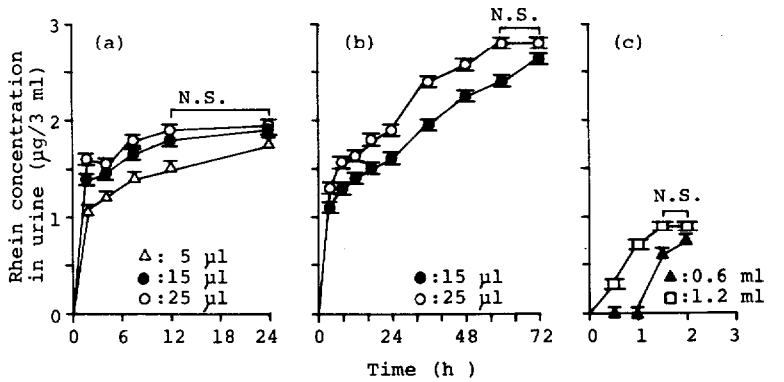

Fig. 2. Time courses of hydrolyses of rhein conjugates in urine obtained after the oral administration of 1 g of powdered senna by β-glucuronidase (100 U/ml) (a), arylsulphatase (25 U/ml) (b) and hydrochloric acid (c). N.S. = no significant difference ($P < 0.05$). Each value represents the mean ± S.D. of three experiments. Conditions of hydrolysis are as described in Experimental.

β-glucuronidase, arylsulphatase and hydrochloric acid are shown in Fig. 2. To ensure complete hydrolysis in urine, the incubation time of each hydrolysis was selected from the results described above. Each chromatogram obtained from urine extracts after hydrolyses by β-glucuronidase, arylsulphatase and hydrochloric acid demonstrated satisfactory resolution.

The present method was applied to the study of normal subjects. The data of urinary excretion of RH and its conjuagates after oral administration of four SL to a normal subject are shown in Table II. The total excretion of RH in urine after administration of one Pursennid tablet was 0.43 mg, corresponding to 5.7% of the given dose.

TABLE II

URINARY EXCRETION OF RHEIN (RH) AND ITS CONJUGATES 24 h AFTER ORAL ADMINISTRATION OF FOUR SENNOSIDE A- AND B-CONTAINING LAXATIVES (SL) TO A NORMAL SUBJECT

SL drug	Dose	Urinary excretion amount as RH (mg)			
		Unchanged form	Hydrolyses		
			β-Glucuronidase	Arylsulphatase	Acid
Prusennid	1 tablet	0.09	0.09	0.14	0.11
Senna	1 g	0.56	0.71	0.99	0.29
Rhubarb	1 g	4.77	0.43	2.69	1.35
Alosenn	1 g	0.27	0.88	0.79	0.31

The urine obtained from a female hypokalemic (cause unknown) patient was analysed by the method of qualitative analysis in a previous paper [6], and RH was detected in her urine. Then, the present method was applied to the urine. The concentration of RH was 0.75 μg/ml and that of RH hydrolysed by β-glucuronidase, arylsulphatase and hydrochloric acid were 0.17, 0.45 and 0.5 μg/ml, respectively. Daily urinary excretion of RH and its conjugates in the female patient could not be estimated, because collection of her urine sample for 24 h was difficult.

In summary, a simple, rapid and precise HPLC method for determination of RH and its conjugates in urine was developed. The present method may be helpful in investigating questions dealing with laxative abuse and disposition of SL.

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