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Note

High-performance liquid chromatographic assay for urinary metabolites of a drug after extraction by antiserum to the drug: an example using *erythro*-9-(2-hydroxy-3-nonyl)hypoxanthine

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erythro-9-(2-Hydroxy-3-nonyl)hypoxanthine (NPT 15392, I, Fig. 1) is an immunopharmacologically active compound [1,2] that is intended for use in the treatment of patients with acquired or genetically determined deficiencies in cell-mediated immune function. Analysis of the drug and its metabolites as required for human pharmacokinetic and metabolic studies posed a challenge because of the low dosage adminstered, approximately 0.01 mg/kg. Determination of the parent drug, I, from human serum and urine samples was accomplished by use of a radioimmunoassay (RIA) [3]. However, development of RIAs for all the metabolites as well would be a large task for the relatively few times such an extensive survey would be required. This paper describes one viable approach to the problem and may find general applicability to the analysis of metabolites of a variety of other drugs for which a RIA is used.

The structure of animal metabolites of I have been determined [4], and include an alcohol [9-(2,8-dihydroxy-3-nonyl)hypoxanthine, II, Fig. 1], the corresponding ketone [*erythro*-9-(2-hydroxy-8-keto-3-nonyl)hypoxanthine, III, Fig. 1], three carboxylic acids [*erythro*-6-hydroxy-5-(9-hypoxanthyl)heptanoic acid, *erythro*-7-hydroxy-6-(9-hypoxanthyl)octanoic acid and *erythro*-8-hydroxy-7-(9-hypoxanthyl)nonanoic acid], as well as glucuronide conjugates of I and II. All the above compounds are highly UV-absorbing, are well retained on reversed-phase columns with various mobile phases and are readily analyzed by high-performance liquid chromatography (HPLC) using a UV detector [4]. However, direct injection of urine on HPLC resulted in overwhelming interferences from normal urine constituents (see Fig. 2a). Pre-treatment of urine samples with

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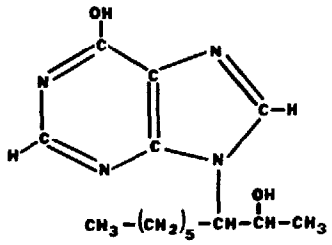
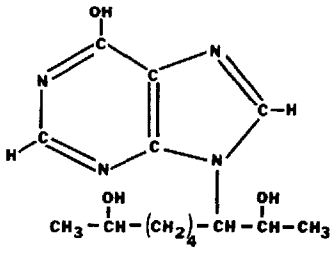
I



II



III

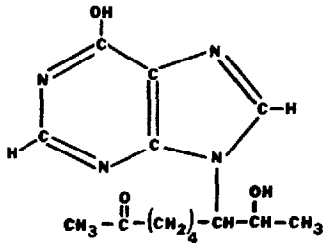


Fig. 1. Structures of *erythro*-9-(2-hydroxy-3-nonyl)hypoxanthine (I), 9-(2,8-dihydroxy-3-nonyl)hypoxanthine (II) and *erythro*-9-(2-hydroxy-8-keto-3-nonyl)hypoxanthine (III).

silica columns, ion-exchange chromatography and organic phase extractions resulted in only limited reduction of interferences.

Sensitivity and excellent selectivity were imparted to the urinary HPLC analysis of II and III using a preliminary rabbit antiserum precipitation. During the development of the RIA for I, antisera from a number of rabbits were collected, one of which exhibited maximal titer, sensitivity and only 1–4% cross-reactivity to various metabolites [3]. The other (unused) rabbit antisera would be expected to also have a similar degree of cross-reactivity and, if added in a large enough excess to the amount of metabolite in urine, could bind to the metabolites and subsequently be precipitated as an antigen–antibody complex. The antigen (metabolite) might then be liberated from the washed precipitate by denaturation of the antibody and analyzed by HPLC. This procedure was in fact successful for metabolites II and III.

EXPERIMENTAL

Materials

Metabolites II and III were isolated as urinary excretion products of I from the rat; estimated final purity was >99% [4]. The glucoronide conjugate of II was

isolated from the urine of the Rhesus monkey after administration of ^{14}C-labeled I. Metabolites II and III were also available radiolabeled with ^{14}C from the previous metabolic study [4]. Antiserum to I was raised in rabbits after subcutaneous injection of bovine serum albumin bonded to I [3]. All HPLC solvents were HPLC-grade except water which was prepared by reverse osmosis, ion exchange and charcoal filtration.

Chromatography

An Altex Model 332 gradient liquid chromatograph (Beckman Instruments, Irvine, CA, U.S.A.), a Waters Model 440 UV detector operating at 254 nm (Waters Assoc., Milford, MA, U.S.A.) and a 250 mm×4.6 mm Ultrasphere 5-μm ODS column water-jacketed at 30°C (Beckman Instruments) were used for HPLC analyses. Sample injection was made using an Altex Model 210 injector using a sample loop of 20 μl. Peak areas were measured with a Hewlett-Packard 3388A digital integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.). The mobile phase used was methanol–water (25:75, v/v), at a flow-rate of 1 ml/min.

Standard curves for each metabolite were determined by duplicate injections of 0, 0.1, 0.5, 1.0, 5.0, 10.0 and 20 μg/ml solutions of II and III in HPLC eluent. Both area responses were linear (correlation coefficient 1.000) and intercepted the y-axis at 22 and 14 ng/ml for II and III, respectively. The response factors for each metabolite were determined on a daily basis by averaging the areas of five determinations of 1 μg/ml standards made up in mobile phase.

Sample preparation

Duplicate aliquots (1.00 ml) of the urine to be analyzed were removed and loaded in a small round-bottom test tube, and to each were added 0.25 ml of 0.5 *M* sodium phosphate pH 7.5, 0.2 ml antiserum (adjusted to 0.6 mg/ml antibody to I) and 0.55 ml water. This mixture was incubated for 2 h at room temperature, then 2.0 ml of saturated ammonium sulfate (SAS) in water was added dropwise, with stirring, to precipitate the rabbit serum proteins and metabolite–antibody complexes. Complete precipitation was achieved after 12 h at 4°C. The sample was centrifuged at 2000 *g* for 20 min in a fixed-angle rotor to make a compact pellet, facilitating removal of the supernatant. The pellet was washed by resuspending in SAS, and repeating the centrifugation and precipitation as above. The pellet was then extracted with 1 ml of 95% ethanol, which liberated the metabolite into the liquid phase, and the sample was centrifuged as before. The supernatant was removed, measured and evaporated to dryness in a small vial. The residue was taken up into 100 μl mobile phase for HPLC analysis. This preparation resulted in approximately a ten-fold concentration of the sample.

The minimum amount of antiserum and SAS necessary for complete precipitation was found by adding increasing amounts of antiserum and SAS to urine samples containing 100 ng/ml ^{14}C-labeled II and III until precipitation was complete as measured by liquid scintillation counting of the precipitates and supernatants. At this level of metabolites, with the addition of 0.2 ml antiserum and 2 ml SAS under the conditions listed above, only 2–5% of the ^{14}C added did not precipitate and remained after washing.

Recovery studies

Control urines were collected from seven untreated individuals. Accurate additions of approximately 10, 25, 50, 100 and 150 ng/ml of II and III were made to each urine sample, and then analyzed as described.

β-Glucuronidase incubations

Conversion of the glucuronide conjugate of II to the unconjugated parent metabolite (II) was carried out by the addition of 110 Fishman units of β-glucuronidase (Type III, *Escherichia coli*, Sigma, St. Louis, MO, U.S.A.) to 1 ml of urine with incubation at 37°C for 3 h. Experiments using a ^{14}C-labeled glucuronide conjugate of II showed quantitative conversion to II by this procedure.

RESULTS

Use of the antibody precipitation for extractions of II and III from urine resulted in the elimination of most interfering compounds normally present. Illustrated in Fig. 2a is an HPLC profile of normal urine injected directly. Illustrated in Fig. 2b and c, respectively, are chromatograms of a patient's urine before and

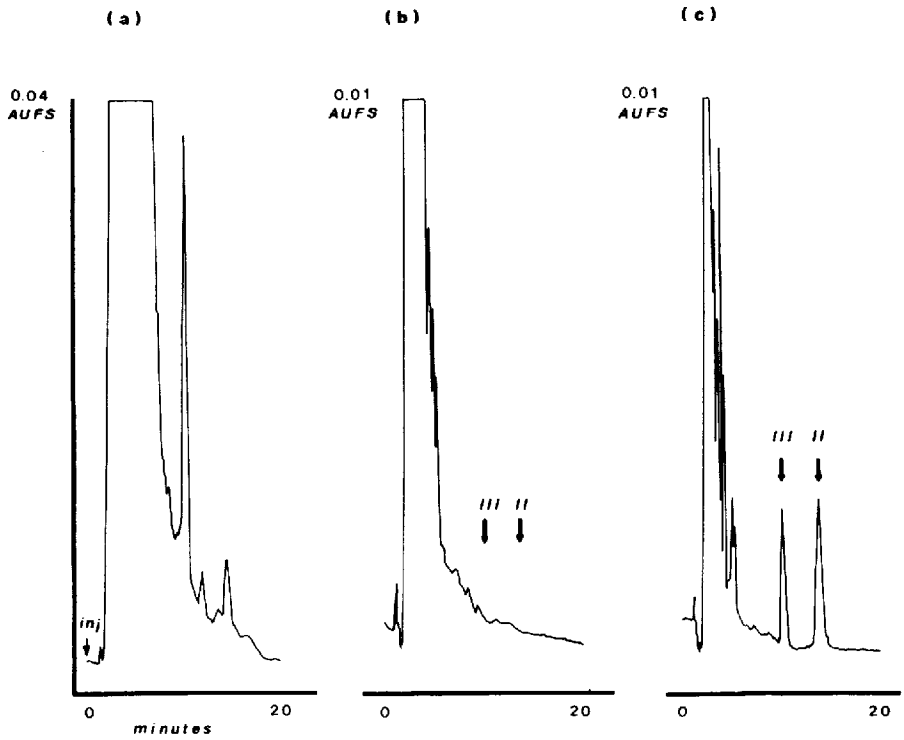


Fig. 2. (a) Chromatogram of a normal human urine analyzed under the conditions as defined in the text; a.u.f.s. = 0.04. (b) Chromatogram of an antiserum extract of urine before treatment; a.u.f.s. = 0.01. (c) Chromatogram of an antiserum extract of the same subject, 2 h after treatment with 0.5 mg I; a.u.f.s. = 0.01.

TABLE I

RECOVERIES OF METABOLITE II FROM NORMAL HUMAN URINE

Added (ng/ml)	Recovered (ng/ml)								Mean recovery (%)	Variance (%)
	A	B	C	D	E	F	G	Mean ± S.D.		
9.93	9.32	8.48	5.66	16.20	11.20	5.16	2.88	8.41 ± 4.43	84.7	53
29.10	32.30	35.20	27.80	42.90	31.30	27.10	24.40	31.6 ± 6.16	109	20
56.10	49.70	56.60	53.90	68.20	58.30	63.40	42.40	56.1 ± 8.55	100	15
110.00	93.30	116.00	96.30	120.00	96.30	137.00	79.20	105 ± 19.7	95.5	19
164.00	-	-	-	149.00	121.00	172.00	120.00	141 ± 24.9	86.0	18

TABLE II

RECOVERIES OF METABOLITE III FROM NORMAL HUMAN URINE

Added (ng/ml)	Recovered (ng/ml)								Mean recovery (%)	Variance (%)
	A	B	C	D	E	F	G	Mean ± S.D.		
8.75	8.68	-	7.74	29.30	-	18.40	6.04	14.0 ± 9.80	160	70
25.70	24.90	32.00	24.90	46.80	26.00	23.90	26.10	29.2 ± 8.19	114	28
49.50	41.40	49.40	54.50	49.00	52.40	46.80	48.30	48.8 ± 4.18	98.6	9
97.00	87.90	102.00	109.00	92.60	103.00	102.00	71.30	95.4 ± 12.7	98.4	13
145.00	-	-	-	130.00	133.00	183.00	115.00	140 ± 29.6	96.6	21

after administration of 0.5 mg of I and the sample treated as described. The UV interference is greatly reduced, even though the sample is concentrated ten-fold, and compounds II and III are easily seen and quantified.

The results of recovery studies for II and III are illustrated in Tables I and II. Recoveries were quantitative over the range studied, although the lowest levels of III at 10 and 25 ng/ml appeared to give high recoveries of 160 and 114%, respectively. These higher values could be explained by the presence of a small interfering peak in the chromatograms of III, particularly in the results from subject D. This peak occurred in the control urine (no addition of metabolite) of subject D to the equivalent extent of 16 ng/ml and in subject F at 4 ng/ml. These interferences are apparently due to dietary influences, since the subjects' food intake was not controlled. Interference due to other drugs was not evaluated since it is expected that patients used in drug metabolism studies will be restricted in this respect.

Variances for the recoveries ranged from 9 to 28% over the concentrations from 26 to 164 ng/ml. Variances at the lowest level, about 10 ng/ml, were considerably higher: 53 and 70%. At this level, 10 ng/ml, the peaks were distinguishable from noise but poorly quantified.

For an initial indication of urinary metabolite levels, two subjects were given 0.5 mg I orally as an aqueous solution and a sample of urine was analyzed at 2 h post administration. Measurable amounts of the metabolites were found, includ-

ing the glucuronide conjugate. Metabolite II was measured at 272 and 119 ng/ml, metabolite III at 146 and 93 ng/ml and the glucuronide conjugate of II at 48 and 16 ng/ml in subjects I and II, respectively. The glucuronide was calculated from the increase in measured levels of II after incubation with β-glucuronidase as described in the Experimental section. Although a single measured value of II was considerably above the recovery levels studied, the original sample of urine may easily be diluted and re-analyzed to bring the concentration within range.

CONCLUSION

A method for the analysis of urinary drug metabolites using a preliminary extraction by antiserum to the parent drug has been illustrated in the case of an experimental compound, *erythro*-9-(2-hydroxy-3-nonyl)hypoxanthine. The method promises to be of general interest in the case of substances for which a RIA has been developed and sufficient antiserum is available. Important requirements for the applicability of the method are: (1) the structure of the metabolite has not been altered to such an extent that the available antiserum does not cross-react to even a few percent; (2) the metabolites bind tightly enough that the antiserum complex can be precipitated and washed; (3) the metabolite of interest can be liberated for analysis; (4) there exists an analytical method for the metabolite after the sample is relatively free of interferences.

Methods other than ethanol denaturation for liberation of the metabolites from the protein precipitate could probably be used in applicable situations. These methods might include enzymatic digestion of the protein or chemical cleavage of the peptide bond. However, denaturation of the antiserum by organic solvents has the advantage that the protein is easily separated from the soluble metabolites by centrifugation.

The accuracy of the illustrated method is sufficient for the intended purpose of demonstrating the appearance of suspected urinary metabolites, as well as a quantitative evaluation at the higher metabolite level. The accuracy could probably be improved by the addition of a radiolabeled internal standard, such as might be available from animal pharmacokinetic studies.

The assay sensitivity is excellent; after administration of less than 1 mg of drug to a subject, the subsequent appearance of metabolites in urine is easily identified and measured. This sensitivity is due to the elimination of interferences from normal human urinary components.

REFERENCES

1 J.W. Hadden and J. Wybran, in J.W. Hadden, L. Chedid, P. Mullen and F. Spreafico (Editors), Advances in Immunopharmacology, Pergamon, New York, 1981, pp. 457-468.
2 S. Ikehara, J.W. Hadden, R. A. Good, D.G. Lunzer and R.N. Pahwa, Thymus, 3 (1981) 87.
3 E.H. Pfadenhauer, C.E. Jones and K.W. Maxwell, J Pharm. Sci., 72 (1983) 914.
4 E.H. Pfadenhauer, C.S. Bankert, J. Jensen, C.E. Jones, E.E. Jenkins and James A. McClosky, Drug Metab. Dispos., 12 (1984) 280.