Journal of Chromatography, 377 (1986) 175—182
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3000

BATCH SEPARATION OF URACIL DERIVATIVES FROM URINE

M. UZIEL

Health and Safety Research Division, Oak Ridge National Laboratory, Building 4500S, P.O. Box X, Oak Ridge, TN 37831 (U.S.A.)

(First received May 24th, 1985; revised manuscript received October 31st, 1985)

SUMMARY

Commercial mixed-bed deionizing resins have been modified by conversion to the sulfonic acid and acetate forms and used to prepare extracts of urine that contain uracil derivatives ($pK_a > 8$). A rapid batch extraction process, requiring less than 15 min is described for the first time that can be used either for rapid spectrophotometric screening of altered levels of these uracil derivatives or for accurate measurement of uracil and pseudouridine by high-performance liquid chromatography on reversed-phase columns using 0.002 *M* ammonium formate (pH 6) in water as the solvent.

The spectrophotometric assay is based on the spectral shift of uracil derivatives when neutral aqueous solutions are made alkaline. After the batch extraction, the factor for conversion of absorbancy change to nmol is 0.0046 per nmol/ml for uracil and 0.0035 per nmol/ml for pseudouridine. Pseudouridine levels in two test urines were found to be 24 and 19.3 nmol/μmol of creatinine, which are consistent with published values of 17.6—24.

INTRODUCTION

Chemical injury can be viewed as the effects of chemical exposure of tissues that lead to cell death and/or non-lethal damage to cellular constituents. In some instances one may expect tissue injury to result in increased levels of urinary excretion of normal end-products of metabolism. The uracil derivatives, viz. thymine, deoxyuridine and pseudouridine, and deoxycytidine, have been observed to exhibit enhanced excretion subsequent to radiation or chemical injury of intact animals or humans [1—4] or cells in culture [5, 6]. With the increased interest in monitoring worker exposure, simple and rapid procedures for extraction of the above uracil derivatives in urine could be valuable screening tools.

The non-ionized character of these uracil derivatives between pH 5 and 7

may be used for selective extraction of these compounds from body fluids. It has been demonstrated that pseudouridine is not retained by either the cation exchanger Dowex 50 [7] or by the anion exchanger Dowex (acetate) 1 × 8 [8]. In addition, Mrochek et al. [8] showed that virtually all the ultraviolet-absorbing organic acids in urine were significantly retained on Dowex acetate 1 × 8 at pH 4.8 . The properties of these resins suggest that a mixed bed of the two resins might permit adsorption of the major ultraviolet-absorbing compounds in urine and leave the neutral uracil derivatives in solution.

While this work was in progress, we became aware of a similar approach used by Evans et al. [9] for extracting uracil derivatives for studies of diseases of the urea cycle. We have confirmed their observation that the neutral uracil derivatives are not adsorbed to the mixed bed, but find significantly lower amounts of pseudouridine in urine. Pseudouridine has been found to be excreted at essentially constant levels in humans [10, 11].

This paper describes the use of commercially available mixed-bed resins to deionize urine and leave neutral uracil derivatives in solution, and also describes additional simple analytical techniques for rapid measurement of uracil and pseudouridine.

EXPERIMENTAL

Resin preparation

Two preparations of mixed-bed resins have been used: Illinois Water Treatment Company cartridge (Rockford, IL, U.S.A.) containing research ion exchanger Model 2 and Bio-Rad AG 501-x8(D) (Richmond, CA, U.S.A.). Both resin mixtures have a blue dye attached to the anion exchanger to signal when the hydroxide has been completely exchanged. The resins were converted to the acetate form with 2 vols. (v/v) of 2 *M* acetic acid with stirring for at least 5 min or longer. The blue dye color may be discharged in this time, but the resin continues to release gas, presumably from adsorbed carbonate. Excess acetic acid is decanted, the resin slurry is transferred to a medium sintered-glass funnel and washed with distilled, deionized water to remove residual acetic acid (final pH of the wash is between 5 and 6.5). The resin is stored as a thick slurry after mixing thoroughly with a plastic scoop. If the resin mixture is washed with sufficient water, there is a partial return of the dye color. This color visually facilitates the remixing of the resins which had separated during the acetate conversion and water-washing steps.

These resin preparations were found to yield urine extracts that had no buffering or acid content, and that could be regularly adjusted to pH 11.5 with a minimum dilution by addition of 0.02 ml of 1 *M* sodium hydroxide to 1.0 ml of extract. These were used in the spectral shift method to quantify uracil and pseudouridine.

Spectrophotometric measurements

Spectrophotometric measurements were made directly on the bead-free extracts using an IBM 9420 UV—VIS spectrophotometer. Supernatant (1 ml) was transferred to the cuvet for measurement of the spectrum between 340 and 230 nm. Changes in absorbance owing to alkaline-induced spectral shifts were

measured after addition of 0.02 ml of fresh 1 *M* sodium hydroxide [12]. The factor for conversion of absorbance change at 262 nm to nmol of compound were obtained by measurement of known amounts of pseudouridine (Fig. 1) and uracil, after treatment with the mixed bed of resins. The absorbance change is the difference in values when measured at neutral pH and pH 11.5. All analyses were carried out in triplicate and averages are reported.

Estimation of yield

Recovery of pseudouridine from urine was determined with a modification of the procedure of Uziel et al. [13] using Affigel 601 as the adsorbent. After adsorption and elution of the urine contaminants, pseudouridine was eluted with 9 ml of 0.1 *M* acetic acid and then analyzed by high-performance liquid chromatography (HPLC).

The volume of resin solids not permeated by water in the batch extraction was measured using the difference between the expected dilution of a stock pseudouridine solution and the observed dilution. Pseudouridine was added to tubes, containing mixed-bed resin and water, in a known volume in order to calculate the dilution factor. Stock pseudouridine solution (0.4 ml) containing 0.174 μmol pseudouridine per ml (absorbance = 1.5) was added to three sets of test tubes. These test tubes (in triplicate) contained either 4.0 ml of water (set a) or 1.8 ml of resin in water in a volume of 4.0 ml (set b) or 3.6 ml of resin in a volume of 4.0 ml (set c). The absorbance at 262 nm was measured and the following equation was used to calculate the resin solids volume x: $4.4\ A = (4.4 - x)\ B$ where A is the absorbance of set a and B are the absorbancies of set b or set c.

Methods for extraction

Two extraction procedures were used. A disposable column approximately 3.5 cm × 1.5 cm (Bio-Rad Catalogue No. 731 1550) was filled with 1.8 ml of resin mixture. The sample was added to the column and followed by elution with water, continuous monitoring at 254 nm and collection of 0.5-ml fractions. In the second procedure, 1.8 ml of resin was placed in a 15-ml centrifuge tube and the sample and water were added so that the total volume was 4.0 ml. After vigorous shaking for about 5–10 s, the beads separate rapidly and samples may be removed immediately for analysis. Turbid urines were also found to be simultaneously clarified and extracted. If the beads cling to the walls, a brief centrifugation will pack them on the bottom of the tube.

All values are the average of at least triplicate analysis.

Absorbance units are defined as the product of absorbance multiplied by the volume of sample at a given wavelength.

HPLC analysis

Samples of the clear supernatant were injected onto a Spherisorb ODS2, 250 mm × 4 mm column (5 μm diameter bead, obtained from ANSPEC, Ann Arbor, MI, U.S.A.) and analyzed by isocratic elution using 0.002 *M* ammonium formate (pH 6). Retention times of 6.7 min (uracil) and 7.1 min (pseudouridine) were obtained at a flow-rate of 1.0 ml/min at room temperature. This analysis could detect 40 pmol of pseudouridine when the recorder was set for

0.04 a.u.f.s. at 254 nm. The compound 5-hydroxymethyluracil coelutes with uracil (retention time 6.7 min). Two compounds not usually found in urine elute at much higher retention times: uridine elutes at 17 min and thymine elutes at 22 min.

Materials

Urine samples used for these studies were either donated by a normal human male or purchased as a lyophilized powder (Ortho control urine I from Ortho Diagnostic Systems, Raritan, NJ, U.S.A.). This material is now sold by Gilford. The lyophilized powder is reconstituted by addition of 25 ml of distilled, deionized water. Fresh human urine was also analysed. Most analyses were done with the commercial urine sample. Affigel 601 and the mixed-bed resin AG501(D) were purchased from Bio-Rad. Pseudouridine, uracil, thymine, uridine, creatinine and cytidine were purchased from Sigma (St. Louis, MO, U.S.A.).

RESULTS

The recovery of pseudouridine, uracil, thymine and uridine from 1.8-ml columns of the mixed-bed resin was measured by continuous monitoring (not shown) of injected samples (0.1, 0.2, 0.4 and 0.6 ml) and by measuring absorbance in 0.5-ml fractions. At 2 ml effluent volume, the recovery after injection of 0.17 to 1.0 absorbance units was 85 ± 5% for each of the compounds, and the recovery was greater than 98% at an effluent volume of 3.5 ml. This indicates that these compounds do not partition into the stationary phase and the volume to elute is determined by the dilution owing to mixing in the column. Cytidine and creatinine were retained by the resin mixture.

The absence of pseudouridine absorption to the resin suggested a simplification of the extraction procedure in which the sample and resin are mixed batchwise and the supernatant is used for analysis. The contribution of the resin solids to the total volume of solution (i.e. not permeated by water)

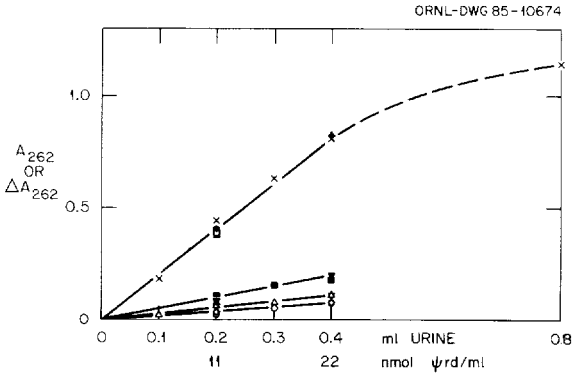

Fig. 1. Absorbance values for extracts of urine and for differential spectral analysis are plotted against volume of urine analyzed or amounts of pseudouridine (Ψrd) analyzed. Two sets of symbols are used: (▲, ▼, △, ◇) batch extraction; (x, o, □, ■, 0) column analysis. The plotted values are urine absorbance at 262 nm (△, x, o) and pseudouridine absorbance at 262 nm (▼, ■); differential absorbance at 262 nm for urine (▽, △) and pseudouridine (◇, 0).

was calculated as described in Experimental where the value of A was 0.136 and the values of B were 0.146 for 1.8 ml of beads and 0.202 for 3.6 ml of beads. The solids accounted for 35% of the volume so that 1.8 ml of resin bed contained 0.65 ml of solid resin.

Fig. 1 shows the absorbance of the column effluent and the supernatant of the batch extraction when both contain 3.5 ml total water volume after sampling 0.1, 0.2, 0.3, 0.4 and 0.8 ml of urine. Both procedures give the same extraction values ($P < 0.025$ predicted range of population mean) for absorbance per ml urine.

The absorbance did not increase proportionately to increased sample size with a 0.8-ml urine sample, indicating the capacity of the exchanger to remove all ionized substances from urine is exceeded above 0.4 ml of urine (Fig. 1). When capacity is exceeded one expects an increased absorbance above the linear value. This decreased response shown in Fig. 1 is not understood but may be expected, since urine does not follow Beer's law until it is diluted 200-fold (not shown). The dilution is only 40-fold or less in these tests, thus the interfering substances may not be adequately removed from a 0.8-ml urine sample.

Analysis of fresh urine also gave a linear increase of absorbance with increased sample volume in the batch extraction procedure (not shown) and a linear increase in differential absorbance measurements from 0.1 to 0.4 ml of applied sample.

The recovery of absorbance change values from the column and batch

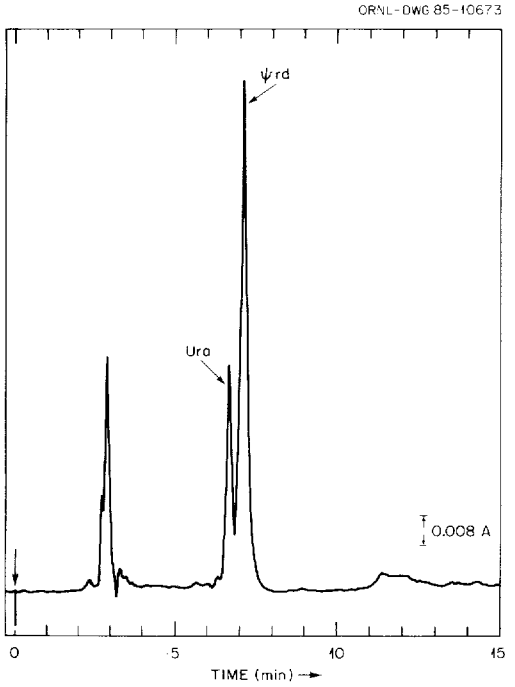

Fig. 2. Chromatographic analysis of urine extract using the batch procedure. The analytical column monitored at 254 nm and solvent are described in Experimental. A 20-μl sample was injected at the arrow, and elution continued at 1 ml/min. Uracil (Ura) eluted at 6.7 ml and pseudouridine (Ψrd) eluted at 7.1 ml.

procedures is also linear with increasing sample size as illustrated in Fig. 1. Application of the spectral shift analysis procedure to these effluents and extracts showed a linear increase of absorbance difference with increasing pseudouridine content (Fig. 1).

Application of the spectral-shift analysis procedure to pseudouridine and uracil extracts from the mixed-bed resin gave the following values for the change in absorbance per nmol per ml: 0.0035 ± 0.0003 (n = 15) for pseudouridine and 0.0046 ± 0.0004 (n = 6) for uracil. Fig. 2 illustrates the chromatographic separation of uracil and pseudouridine from a typical urine extraction. Table I illustrates the recovery of pseudouridine from urine by batch extraction and by procedures demonstrated to quantitatively extract and measure

TABLE I

RECOVERY OF PSEUDOURIDINE AND URACIL FROM URINE

Two types of recovery are listed: the recovery of change in absorbancy at 262 nm owing to the spectral shift in alkali and the recovery of pseudouridine. The values reported are the total change in absorbance units (absorbance × volume in ml) and concentrations (± 5%) in urine (nmol/ml).

Procedure*	Change in absorbance at 262 nm	Concentration (nmol/ml)	
		Pseudouridine	Uracil
MB	0.963		
MB + HPLC	0.990**	191**	70**
Boronate + HPLC		201	
Recovery (%)	103	95	

*The procedure MB is the batch extraction of urine. HPLC is as described in Experimental. Boronate is the affinity chromatographic procedure using Affigel 601 that gives total pseudouridine in urine [13] (see Experimental).

**These values were calculated by conversion of the nmol of pseudouridine and uracil to the expected absorbance change in the alkaline spectral shift assay using the conversion factors of 0.0035 per nmol pseudouridine and 0.0046 per nmol uracil.

TABLE II

RECOVERY OF ABSORBANCE USING BATCH EXTRACTION

Three sets of triplicate analyses were used to test the recovery of pseudouridine solutions added to urine. The stock urine and pseudouridine solutions were tested separately using two volumes (0.2 and 0.4 ml) of each solution. The sum is obtained by addition of the two values in the column. The "observed" value is obtained by mixing equal volumes of urine and pseudouridine and then analyzing 0.4 and 0.8 ml of the mixture. The higher volumes will contain the same total amount of compounds as the individual tests.

	Absorbance at 262 nm		Change in absorbance at 262 nm	
	0.2 ml	0.4 ml	0.2 ml	0.4 ml
Urine	0.41 ± 0.04	0.82 ± 0.04	0.06 ± 0.01	0.11 ± 0.01
Pseudouridine	0.10 ± 0.03	0.20 ± 0.02	0.04 ± 0.01	0.08 ± 0.01
Sum	0.51	1.02	0.10	0.19
Observed	0.56 ± 0.03	0.93 ± 0.05	0.12 ± 0.02	0.17 ± 0.02

pseudouridine [13]. The batch extraction gave 95% of the reference value. Since no comparable procedure exists for uracil we have estimated its recovery indirectly. The alkaline shift data are the sum of absorbance changes for both pseudouridine and uracil. We calculated the amount of absorbance change expected using the measured conversion factors of 0.0035 for pseudouridine and 0.0046 for uracil. The calculated recovery was 103%. As an additional check, we added pseudouridine to urine and then measured recoveries using the alkaline shift procedure. Table II illustrates the quantitative recovery. Table II and Fig. 1 show the extraction of uracil and pseudouridine by alkaline shift and by HPLC.

The concentrations of pseudouridine found by HPLC analysis of the batch extracts were 195 μmol/l for commercial urine (pregnant females) and 363 μmol/l for fresh human urine (male, age 55 years). Creatinine measurements showed concentrations of 7.9 mmol/l for the commercial urine (obtained from pregnant women) and 18.4 mmol/l for the human male. These yielded ratios of nmol of pseudouridine to μmol of creatinine of 24.6 for the commercial urine and 19.7 for the normal male urine. Published values of nmol of pseudouridine per μmol creatinine for studies of normal humans have been reported as 22.4 ± 8% (S.D.) [10], 22.4 ± 10% [4], 22.6 ± 20% [11], and 24 (no S.D. given) [14]. Evans et al. [9] however, reported values of 44 nmol pseudouridine per μmol creatinine (calculated from data of Table III of ref. 9).

DISCUSSION

The challenge in measurement of pseudouridine is separation of the compound from its complex environment, whether in RNA or in body fluids. Assay sensitivity (40 pmol) is not always a significant challenge, since the substance is usually present in relatively high concentration: about 0.7% of mixed RNA, 0.2 mmol/l in urine [15] and about 2 μmol/l in serum [16].

Pseudouridine in urine has been quantitatively measured by combined chromatography and absorbance measurements, combined affinity chromatography and absorbance measurements [13], competitive binding to radiolabeled haptene- antibody complex [17], and now combined batch extraction and HPLC. The differential spectral analysis does not distinguish between uracil and pseudouridine but may still be useful for screening for changes in excretion levels. Each of these procedures combines physical separation (selective binding to protein or chromatographic discrimination) and selective detection procedures (absorbance, radioactivity or differential absorbance measurements) to measure the neutral uracil derivatives. With this variety of procedures, the selection of one for a given study will depend on factors other than separation method or sensitivity.

The primary advantages of the procedures described in this study are speed and low cost: the time from dispensing the resin mixture to completion of HPLC for a single analysis is less than 15 min; the costs of reagents are minimal since the resins can be regenerated and the HPLC solvent is essentially water. Thus the procedures described here will be advantageous when time and cost are a primary concern.

There are two potential applications for the combined urine deionizing

and differential spectral analysis where time and cost may be of concern: measurements of chemical injury (e.g. monitoring worker exposures) where neutral uracil derivatives are excreted in increased amounts [1- 3], and measurements of pseudouridine excretion during chemotherapy. Studies have shown that cancer patients excrete increased levels of pseudouridine [18], and that the return of pseudouridine levels to normal values is a valuable indicator of remission of cancer [4, 14, 19]. Application of this approach to older patients (60- 90 years) may require special attention since their pseudouridine/creatinine levels have been reported to be almost twice the value of 20- 55-year-old normals [20]. The differential spectral analysis would provide data for rapid screening of urine extracts for enhanced excretion of neutral uracil derivatives, and HPLC would provide the exact amounts of each uracil derivative in the mixture.

ACKNOWLEDGEMENTS

Research was sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract No. DE-AC05-840R21400 with Martin Marietta Energy Systems.

Joan Adams provided valuable assistance in this project. Funds for her support came from the Technology Internship Program with State Technical Institute of Knoxville that is administered by Oak Ridge Associated Universities.

REFERENCES

1 J.R. Rubini, E.P. Cronkite, V.R. Bond and T.M. Fliedner, Proc. Soc. Exp. Biol., 100 (1958) 130.
2 J. Parizek, M. Arient, Z. Dienstbier and J. Skoda, Nature, 182 (1958) 721.
3 L.O. Froholm, Int. J. Rad. Biol., 12 (1967) 35.
4 E. Borek, O.K. Sharma and T.P. Waalkes, Modified Nucleosides and Cancer, Springer, New York, 1983, p. 301.
5 M. Uziel, B. Haas and J.K. Selkirk, Chemical Analysis and Biological Fate: Polynuclear Aromatic Hydrocarbons, Battelle Press, Columbus, OH, 1981, p. 687.
6 M. Uziel, B. Owen and A. Butler, J. Appl. Toxicol., 6 (1986) in press.
7 M. Uziel, C. Koh and W.E. Cohn, Anal. Biochem. 25 (1968) 77.
8 J.E. Mrochek, W.C. Butts, W.T. Rainey, Jr. and C.A. Burtis, Clin. Chem., 17 (1971) 72.
9 J.E. Evans, H. Tieckelmann, E.W. Naylor and R. Guthrie, J. Chromatogr., 163 (1979) 29.
10 M. Uziel and S.A. Taylor, J. Carbohydr. Nucleosides Nucleotides, 5 (1978) 235.
11 C. Gehrke, K.C. Kuo, T.P. Waalkes and E. Borek, Cancer Res., 39 (1979) 1150.
12 W.E. Cohn, J. Biol. Chem., 235 (1960) 1488.
13 M. Uziel, L.H. Smith and S.A. Taylor, Clin. Chem., 22 (1976) 1451.
14 F. Salvatore, A. Colonna, F. Costanzo, T. Russo, F. Esposito and F. Cimmino, Modified Nucleosides and Cancer, Springer, New York, 1983, p. 360.
15 G.B. Chheda, Handbook of Biochemistry, The Chemical Rubber Company, Cleveland, OH, 2nd ed., 1971, p. G106.
16 L. Levine, T.P. Waalkes and L. Stolbach, J. Natl. Cancer Inst., 54 (1975) 341.
17 L. Levine and H. Gjika, Arch. Biochem. Biophys., 164 (1974) 583.
18 T.P. Waalkes, C.W. Gehrke, R.W. Zumwalt, S.Y. Chang, D.B. Lakings, D.C. Tormey, D.L. Ahmann and C.G. Moertel, Cancer, 36 (1975) 390.
19 T. Rasmuson, G.R. Bjork, L. Damber, S.E. Holm, L. Jacobson, A. Jeppsson, B. Littbrand, T. Stigbrand and G. Westman, Modified Nucleosides and Cancer, Springer, New York, 1983, p. 331.
20 G.L. Tritsch, J.M. Luch, J.T. Evans and A. Mittelman, Biochem. Med., 22 (1979) 387-390.