Journal of Chromatography, 515 (1990) 495–501
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1752

Determination of pseudouridine in human urine and serum by high-performance liquid chromatography with post-column fluorescence derivatization

YOSHIHIKO UMEGAE, HITOSHI NOHTA and YOSUKE OHKURA*
Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

ABSTRACT

A selective and sensitive method for the determination of psuedouridine in human urine and serum is described. The method is based on high-performance liquid chromatography with post-column fluorescence derivatization. Pseudouridine and 5-fluorouridine (internal standard) in a 10-fold diluted urine sample or a deproteinized serum sample are separated on a reversed-phase column (TSK gel ODS-80_{TM}) with isocratic elution and successively subjected to derivatization involving periodate oxidation followed by fluorescence reaction with *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine. The detection limit for pseudouridine is 4 pmol in a 100-μl injection volume.

INTRODUCTION

Urinary excretion of modified nucleosides which result from the enzymatic degradation of ribonuleic acid (RNA), especially from transfer-RNA, has been found to increase in a variety of neoplastic diseases[1–4], and pseudouridine is a nucleoside that increases most frequently[4–7]. Therefore, pseudouridine in urine can be utilized as a tumour marker for the diagnosis and follow-up of the diseases. Pseudouridine in serum has also been measured[6,8,9].

Most of currently available methods for the determination of pseudouridine are based on liquid chromatography with UV detection. Although these methods are fairly sensitive, they are not very selective and thus require complicated clean-up using boronate gel chromatography[2–8] or high-performance liquid chromatography (HPLC) on two reversed-phased columns[10].

In a previous paper[11], we reported that 1,2-bis(4-methoxyphenyl)ethylenediamine (p-MOED) reacted sensitively and selectively with ribonucleosides and ribonucleotides in an acidic medium after periodate oxidation to produce fluorescent derivatives.

The purpose of this study was to establish a highly sensitive and selective HPLC method for the determination of pseudouridine in human urine and serum, based on

0021-9673/90/$03.50 © 1990 Elsevier Science Publishers B.V.

HPLC separation on a reversed-phased column followed by post-column derivatization utilizing the above-mentioned fluorescence reaction. 5-Fluorouridine was used as an internal standard.

EXPERIMENTAL

Reagents and solutions

p-MOED was synthesized as described previously[12]. Pseudouridine and 5-fluorouridine were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical-reagent grade. Deionized, distilled water was used.

Urine and serum samples were obtained from healthy subjects in the absence of preservative or anticoagulant, and kept frozen at −20°C until used. Sodium phosphate buffers (10 m*M*, pH 5.0) containing 4 and 2% (v/v) of methanol were used as HPLC mobile phases for urine and serum samples, respectively. p-MOED solution (20 m*M*) was prepared in 0.14 *M* perchloric acid containing 68% (v/v) of ethanol. Sodium periodate solution (2 m*M*) was prepared in water. Both p-MOED and sodium periodate solutions were used for fluorescence derivatization.

HPLC system and its operation

Fig. 1 shows a schematic diagram of the HPLC system constructed for the determination of pseudouridine. A 100-μl aliquot of sample solution was injected into a CCPM chromatograph (Tosoh, Tokyo, Japan) equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a TSK gel ODS-80_{TM} column (particle size 5 μm; 150 × 4.6 mm I.D.) (Tosoh). The mobile phase was pumped at a flow-rate of 0.5 ml/min. The column temperature was ambient (25 ± 2°C). The column eluate was first passed through a Tosoh UV-8010 detector (254 nm; flow cell, 10.7 μl) if it was required to monitor the absorbance in comparison with fluorescence

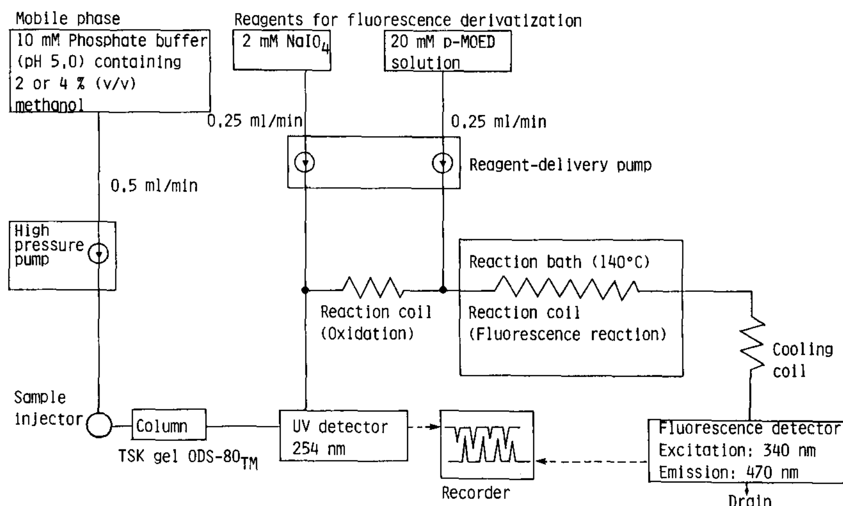

Fig. 1. Schematic diagram of the HPLC system.

detection. Sodium periodate solution was then added to the eluate stream by means of an SSP PM 1024 pump (Sanuki Kogyo, Tokyo, Japan) at a flow-rate of 0.25 ml/min, and the mixture was passed through a reaction coil (1 m × 0.5 mm I.D. stainless-steel tube) for oxidation. The p-MOED solution was added to the stream at a flow-rate of 0.25 ml/min. The mixture was then heated in a reaction coil (20 m × 0.5 mm I.D. stainless-steel tube) placed in a Shimadzu (Tokyo, Japan) CRB-6A reaction bath (140°C) to develop the fluorescence and the reaction mixture was passed through an air-cooling coil (1 m × 0.25 mm I.D. stainless-steel tube). The fluorescence intensity of the last effluent was monitored at 470 nm emission with excitation at 340 nm (both spectral band widths 20 nm) using a Tosoh FS-8000 spectrofluorimeter equipped with a flow cell (15 μl). Peak height was used for quantification.

HPLC sample preparation

Urine sample. To 100 μl of urine, 100 μl of 500 nmol/ml 5-fluorouridine (internal standard) and 800 μl of water were added. A 100-μl aliquot of the mixture was used for HPLC. Urinary creatinine was measured by using a creatinine test kit (Wako, Osaka, Japan).

Serum sample. To 0.5 ml of serum were added 0.5 ml each of 50 nmol/ml 5-fluorouridine solution and 2.0 *M* perchloric acid and the mixture was centrifuged at 1000 *g* for 10 min at 4°C. To 0.5 ml of the supernatant were added 65 μl of 2 *M* potassium carbonate solution and the mixture was briefly centrifuged. A 100-μl aliquot of the supernatant was subjected to HPLC.

RESULTS AND DISCUSSION

HPLC conditions

Fig. 2 shows a chromatogram obtained by fluorescence detection with a standard mixture of pseudouridine and 5-fluorouridine using sodium phosphate buffer (10 m*M*, pH 5.0) containing 4% (v/v) methanol as the mobile phase (retention times

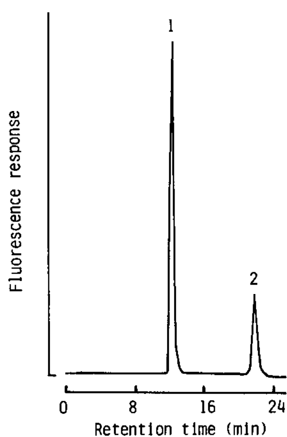

Fig. 2. Chromatogram obtained with a standard mixture of pseudouridine and 5-fluorouridine (fluorescence detection). Mobile phase: 10 m*M* sodium phosphate buffer (pH 5.0) containing 4% (v/v) of methanol. Peaks: 1 = pseudouridine; 2 = 5-fluorouridine. Concentrations: 2.0 nmol/ml each.

12.0 and 21.6 min, respectively). The eluates from peaks 1 and 2 both have fluorescence excitation and emission maxima at 340 and 470 nm, respectively.

The retention times of pseudouridine and 5-fluorourdine decreased with increasing concentration of methanol in the mobile phase. At a concentration greater than 5% (v/v) the pseudouridine peak overlapped with an early eluting large peak for unknown substance(s) in urine or serum (see Figs. 3A and 4A). In the absence of methanol, much longer times were required for the elution of pseudouridine and 5-fluorouridine (retention times 16.0 and 34.0 min, respectively). Methanol concentrations of 4 and 2% (v/v) were the most satisfactory for urine and serum samples, respectively. Although the sodium phosphate concentration only slightly affected the retention times in the range 10–100 m*M*, the background fluorescence intensity increased with increasing concentration of phosphate; 10 m*M* gave the highest signal-to-noise ratio. The pH of 10 m*M* sodium phosphate buffer had no effect on the retention times of the peaks in the range 3.0–7.0; pH 5.0 was employed.

Post-column fluorescence derivatization

Sodium periodate concentrations ranging from 1 to 3 m*M* yielded the highest peaks for pseudouridine and 5-fluorouridine; the selected concentration of 2 m*M* gave reproducible results. Perchloric acid, which was the acid required for the fluorescence reaction, provided almost maximum peak heights for both compounds in the concentration range 0.05–0.15 *M* in the p-MOED solution; 0.14 *M* was used. Water-miscible organic solvents such as methanol, ethanol and acetonitrile served to accelerate the fluorescence reaction. Of these solvents, ethanol was most effective in the concentration range 60–80% (v/v) in the p-MOED solution; 68% (v/v) was selected as optimum. The highest peaks were attained at p-MOED concentrations of 15–25 m*M*; 20 m*M* was employed.

A reaction coil (0.5 mm I.D.) length of 1 m for the oxidation, was the most satisfactory. Almost maximum and constant peak heights were achieved when a reaction coil (0.5 mm I.D.) of 15–25 m was used for the fluorescence reaction; a 20-m reaction coil was recommended in the HPLC system. Higher temperatures allowed the peak heights to increase more rapidly up to *ca.* 150°C. A temperature higher than 150°C caused background noise in the chromatogram owing to gas-bubble formation in the reaction coil; 140°C was optimum.

Determination of pseudouridine in urine and serum

5-Fluorouridine, an artificial nucleoside, was employed as an internal standard. Fig. 3 depicts typical chromatograms obtained with human urine. Chromatogram A, obtained with fluorescence detection, is much simpler than B, obtained with UV detection. This indicates that fluorescence detection is fairly selective for the nucleosides. Although urine contains diverse modified nucleosides (other than pseudouridine) that could react with p-MOED to afford fluorescent compounds, they did not interfere with the assay of pseudouridine because their concentrations were much lower than that of pseudouridine. The peak component pseudouridine was identified on the basis of the retention time in comparison with the standard solution and also by co-chromatography of the standard and the samples.

Fig. 4 shows typical chromatograms obtained for human serum with fluorescence and UV detection. The recommended deproteinization procedure, which used

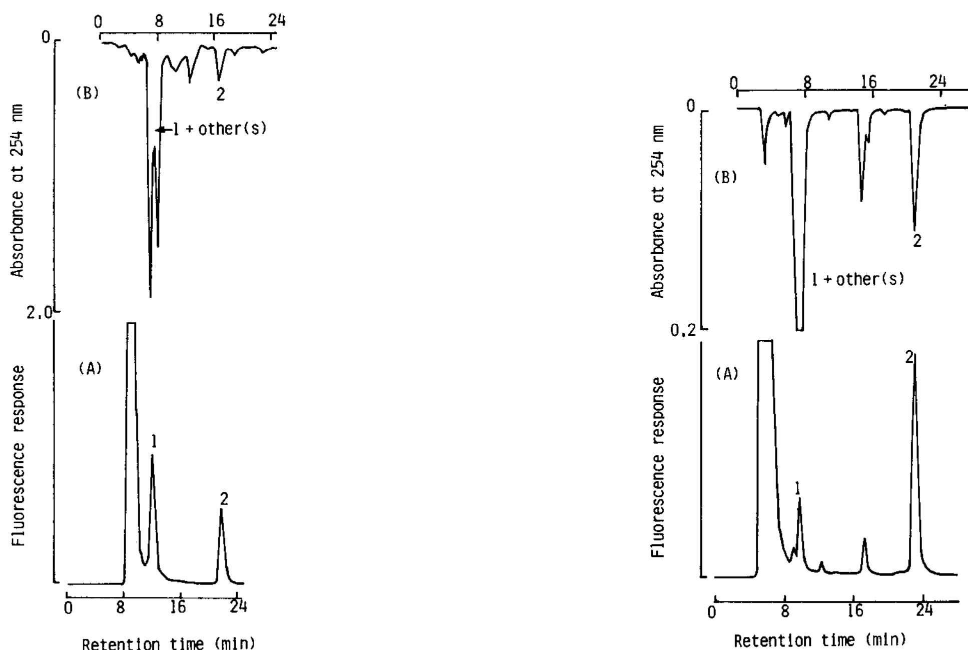

Fig. 3. Chromatograms obtained with (A) fluorescence and (b) UV detection in the HPLC of pseudouridine in human urine. Mobile phase: 10 m*M* sodium phosphate buffer (pH 5.0) containing 4% (v/v) of methanol. Peaks: 1 = pseudouridine (238 nmol/ml); 2 = 5-fluorouridine (internal standard) (500 nmol/ml); others = unidentified.

Fig. 4. Chromatograms obtained with (A) fluorescence and (B) UV detection in the HPLC of pseudouridine in human serum. Mobile phase: 10 m*M* sodium phosphate buffer (pH 5.0) containing 2% (v/v) of methanol. Peaks: 1 = pseudouridine (2.50 nmol/ml); 2 = 5-fluorouridine (internal standard) (50.0 nmol/ml); others = unidentified.

TABLE I

URINARY EXCRETION OF PSEUDOURIDINE FROM HEALTHY PERSONS

Sex[a]	*Age (years)*	*Concentration*[b] *(nmol/μmol creatinine)*
M	42	19.2
M	31	17.0
F	28	27.1
F	26	23.2
M	25	19.5
M	24	16.3
M	23	19.2
F	23	20.4
F	22	26.6
F	21	19.7
	Mean ± S.D.	20.8 ± 3.5

[a] M = Male; F = female.
[b] Measured using 24-h urine samples.

TABLE II

CONCENTRATIONS OF PSEUDOURIDINE IN SERA FROM HEALTHY PERSONS

Sex	*Age (years)*	*Concentration (nmol/ml)*
M	59	2.93
M	32	1.84
M	28	2.02
M	26	2.01
M	26	2.50
M	26	1.78
M	23	1.65
F	26	1.64
F	24	1.20
F	21	2.50
	Mean ± S.D.	2.01 ± 0.48

perchloric acid, provided complete recoveries (99 ± 2%; mean ± relative standard deviation, $n = 5$) of pseudouridine asnd 5-fluorouridine added to 500 μl of serum sample in amounts of 4 nmol each. Other deproteinization methods (trichloroacetic acid, acetonitrile and ultrafiltration through a Millipore Ultrafree C3TK membrane) gave poor recoveries.

Linear relationships were observed between the amounts (y, nmol) of pseudouridine obtained by the internal standard method and the amounts (x, nmol) added in the range 0.5–50 nmol to 100 μl of urine ($y = 1.002x - 0.17$, $r = 0.999$) and 0.1–4 nmol to 0.5 ml of serum ($y = 1.003x - 0.06$, $r = 0.998$). The limits of detection (signal-to-noise ratio = 3) for pseudouridine in urine and serum were 40 and 8 pmol/ml (both corresponded to 4 pmol per 100-μl injection volume), respectively. The relative standard deviations in replicate determinations ($n = 10$) of pseudouridine were 2.2 and 2.7% at mean concentrations of 233 and 1.6 nmol/ml in urine and serum, respectively.

The amounts of pseudouridine in urine and serum from healthy persons assayed by this method are given in Tables I and II, respectively. The values are in good agreement with previously reported data[8–10].

This method is sensitive and so selective that it does not require complicated clean-up procedures. Therefore, this method should be useful for the diagnosis and follow-up of neoplastic diseases and for the biomedical investigation of modified nucleosides.

REFERENCES

1 T. P. Waalkes, C. W. Gehrke, R. W. Zumwalt, S. T. Chang, D. B. Lakings, D. C. Tormey, D. L. Ahmann and C. G. Moertel, *Cancer*, 36 (1975) 390.
2 T. P. Waalkes, M. D. Aeloff, D. S. Ettinger, K. B. Woo, C. W. Gehrke, K. C. Kuo and E. Borek, *Cancer*, 50 (1982) 2457.
3 S. Tamura, Y. Amuro, T. Nakano, J. Fujii, Y. Moriwaki, T. Yamamoto, T. Hada and K. Higashino, *Cancer*, 57 (1986) 1571.
4 J. Speer, C. W. Gehrke, K. C. Kuo, T. P. Waalkes and E. Borek, *Cancer*, 44 (1979) 2120.

5 K. C. Kuo, C. W. Gehrke, R. A. McCune, T. P. Waalkes and E. Borek, *J. Chromatogr.*, 145 (1978) 383.
6 T. Russo, A. Colonna, F. Salvatore, F. Cimiso, S. Brides and C. Gurge, *Cancer Res.*, 44 (1984) 2567.
7 S. Tamura, J. Fujii, T. Nakano, T. Hada and K. Higashino, *Clin. Chim. Acta,* 154 (1986) 125.
8 A. Colonna, T. Russo, F. Esposito, F. Salvatore and F. Cimino, *Anal. Biochem.*, 130 (1983) 19.
9 L. Levine, T. P. Waalkes and L. Stolbach, *J. Natl. Cancer Inst.*, 54 (1975) 341.
10 T. Yamamoto, K. Higashino, S. Tamura, H. Fujioka, Y. Amuro and T. Hada, *Anal. Biochem.*, 170 (1988) 387.
11 Y. Umegae, H. Nohta and Y. Ohkura, *Chem. Pharm. Bull.*, 38 (1989) 452.
12 F. Vogtle and E. Goldshmitt, *Chem. Ber.*, 109 (1976) 1.