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Determination of urinary glucuronide conjugates by high-performance liquid chromatography with pre-column fluorescence derivatization

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Abstract

A simple and highly sensitive high-performance liquid chromatographic method for the direct determination of urinary glucuronide conjugates is described. The method is based on the direct derivatization of the glucuronic acid moiety in glucuronide conjugates with 6,7-dimethoxy-1-methyl-2 (1 H)-quinoxalinone-3-propionylcarboxylic acid hydrazide. The derivatization reaction proceeds in aqueous solution in the presence of pyridine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 0–37°C. The resulting fluorescent derivatives are separated on a C_{18} column using methanol–acetonitrile–0.5% triethylamine in water (1:1:2, v/v) as mobile phase, and are detected spectrofluorimetrically at 445 nm with excitation at 367 nm. The detection limits (signal-to-noise ratio = 3) for the glucuronides are 13–48 fmol for an injection volume of 10 μl (130–480 fmol per 5 μl of human urine). The method was applied to the measurement of etiocholanorone-3-glucuronide and androsterone-3-glucuronide in human urine. The method is simple and rapid without conventional liquid–liquid extraction of the glucuronides from urine.

1. Introduction

Many biochemically and clinically important endogenous and foreign substances are metabolized in the liver to produce mainly their glucuronide conjugates. The resulting polar and thermally labile conjugates are excreted in the urine. The measurement of these glucuronide conjugates gives important information as to their metabolism and is of considerable interest in biological and metabolic studies.

Many high-performance liquid chromatographic (HPLC) methods have been developed for the determination of glucuronide conjugates. Most of these methods are based on the measurement of their aglycon released before and after hydrolysis of the conjugates. Therefore, these conventional indirect methods lack specificity and errors may occur because of incomplete hydrolysis or loss of aglycon. Recently, a spectrophotometric HPLC method based on the direct derivatization of the glucuronic acid moiety of the conjugates with 4-bromomethyl-7-methoxycoumarin has been developed to overcome this problem [1]. However, this reagent is not very sensitive and requires dried aprotic solvents and long heating at high temperature (70°C) for the derivatization. Therefore, this method is not ideal for the determination of the

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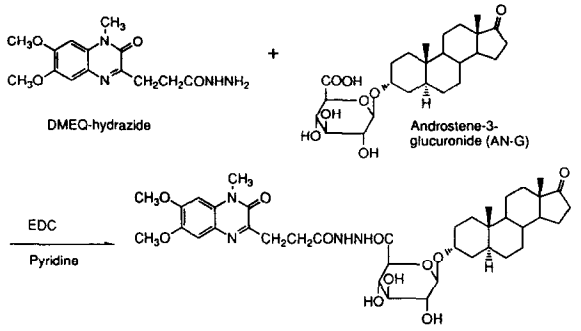

Fig. 1. Fluorescence derivatization of androsterone-3-glucuronide with DMEQ-hydrazide.

polar and thermally-labile glucuronide conjugates.

We developed 6,7-dimethoxy-1-methyl-2(1 H)-quinoxalinone-3-propionylcarboxylic acid hydrazide (DMEQ-hydrazide) as a sensitive and selective fluorescence derivatization reagent for carboxylic acids [2]. The reagent reacted with carboxylic acids even in aqueous solution using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as a coupling agent under mild temperature. Thus, the reagent was successfully applied to the direct determination of free fatty acids in human plasma [3].

Recently, we found that DMEQ-hydrazide also reacts with the carboxyl group of glucuronic acid of the conjugates (Fig. 1). In this paper, we describe an HPLC method with fluorescence detection for the determination of glucuronide conjugates with DMEQ-hydrazide. In order to investigate the reactivity of DMEQ-hydrazide with glucuronides, we used estrone-3-glucuronide (ES-G), testosterone-3-glucuronide (Ts-G), dehydroisoandrosterone-3-glucuronide (DHEA-G), etiocholanorone-3-glucuronide (ETIO-G), and androsterone-3-glucuronide (AN-G) as model compounds of glucuronide conjugates. The method was applied to the determination of ETIO-G and AN-G in human urine by direct derivatization.

2. Experimental

2.1. Reagents and chemicals

All chemicals were of analytical-reagent grade, unless noted otherwise. The glucuronides listed in Table 1 were all obtained from Sigma (St. Louis, MO, USA). De-ionized and distilled water, purified with a Milli-Q II (Millipore, Milford, MA, USA) system, was used for all aqueous solutions. DMEQ-hydrazide was prepared as described previously [2]; it is now commercially available from Wako Pure Chemicals (Tokyo, Japan). DMEQ-hydrazide solutions were prepared in N,N-dimethylformamide and could be used for at least 2 weeks. DMEQ-hydrazide solutions (10 m*M* and 50 m*M*) were used for the derivatization of the glucuronides in the standard solutions and urine samples, respectively. A pyridine (4%) solution was prepared in 20 m*M* hydrochloric acid ethanolic solution; the ethanolic solution was prepared by dissolving 1 vol. of 1.2 *M* hydrochloric acid in water with 60 vol. of ethanol. EDC (4.0 *M*) solution was prepared in water.

Stock solutions of the glucuronides ($1.0 \cdot 10^{-4}$ *M*) were prepared in water and stored at −20°C until use. The solutions were diluted further with water to the desired concentrations before use.

2.2. Samples

Urine samples (24 h) were collected from healthy volunteers in our laboratories. No preservatives were added and the urine samples were frozen on dry-ice immediately after collection.

2.3. Derivatization procedure

To 5 μl of an aqueous test solution (or urine sample) were added 5 μl of water (or glucuronide standard solutions) and 50 μl of the pyridine solution. The mixture was vortex-mixed for *ca.* 10 s and then 25 μl of the DMEQ-hydrazide solution and 15 μl of the EDC solution were added. The resulting mixture was warmed at 37°C for 20 min. The reaction mixture (10 μl) was injected onto the chromatograph. For the reagent blank, 5 μl of water instead of 5 μl of a test solution was subjected to the same procedure.

Table 1
Retention times and detection limits for DMEQ-derivatives of glucuronide conjugates and other substances

Compound	Retention time (min)	Detection limit (fmol/10 μl)	Mobile phase[a]
1,3,5[10]-Estratrien-3-ol-17-one-3-glucuronide (estrone-3-glucuronide)	8.3	15.8	A
4-Androsten-17β-ol-3-one-17-glucuronide (testosterone-17-glucuronide)	10.9	14.6	A
5-Androsten-3β-ol-17-one-3-glucuronide (dehydroisoandrosterone-3-glucuronide)	12.5	12.4	A
Etiocholan-3α-ol-17-one-3-glucuronide (etiocholanorone-3-glucuronide)	30.3	29.5	A
5α-Androsten-3α-ol-17-one-3-glucuronide (androsterone-3-glucuronide)	31.7	33.9	A
1,3,5[10]-Estratriene-3,17β-diol-3-glucuronide (17β-estradiol-3-glucuronide)	6.2	13.6	B
1,3,5[10]-Estratriene-3,17β-diol-17-glucuronide (β-estradiol-17-glucuronide)	7.9	13.8	B
1,3,5[10]-Estratriene-3,16α,17β-triol-3-glucuronide (estriol-3-glucuronide)	11.2	21.0	B
1,3,5[10]-Estratriene-3,17β-diol-3-glucuronide-17-sulfate (17β-estradiol-3-glucuronide-17-sulfate)	16.6	48.7	B
20β-Carboxy-11-oxo-30-norolean-12-en-3β-yl-2-O-β-D-glucopyranuronosyl-α-D-glucopyranosiduronic acid (glycyrrhizin)	14.2	25.8	B
1,3,5[10]-Estratriene-3,16α,17β-triol-3-glucuronide (estriol-17β-glucuronide)	12.4	31.6	C
Glucuronic acid	24.3	105	D
Galacturonic acid	24.3	105	D

[a] Mobile phase = A: methanol–acetonitrile–0.5% triethylamine (1:1:2, v/v); B, C and D: methanol–0.5% triethylamine (methanol concentration: 40, 42 and 20%, respectively).

The amounts of ETIO-G and AN-G in human urine were calibrated by means of the standard addition method: the 5 μl of water added to the urine sample in the derivatization procedure was replaced by 5 μl of the glucuronide standard solutions. The net peak heights of the individual glucuronides were plotted against the concentrations of the spiked glucuronides.

2.4. Chromatography

A Hitachi L-6200 high-performance liquid chromatograph (Tokyo, Japan) equipped with a high-pressure sample injector (20-μl loop) and a Jasco FP-210 spectromonitor (Tokyo, Japan) fitted with a 15-μl flow-cell were used. It was operated at an excitation wavelength of 367 nm and an emission wavelength of 445 nm. The DMEQ derivatives of the glucuronide conjugates were separated on a L-column ODS column (250 × 4.6 mm I.D., particle size 5 μm) (Chemicals Inspection and Testing Institute, Tokyo, Japan), fitted with a guard column (TSK gel ODS-120T, 10 × 4 mm I.D., particle size 5 μm) (Tosoh, Tokyo, Japan), by isocratic elution with methanol–acetonitrile–0.5% triethylamine in water (1:1:2, v/v). The flow-rate of the mobile phase was 1.0 ml/min. The column temperature was maintained at 40 ± 0.2°C with a Shimadzu CTO-6A column oven (Kyoto, Japan). Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow-cell; the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

3. Results and discussion

3.1. Separation of DMEQ derivatives of glucuronide conjugates

The simultaneous separation of five glucuronide conjugates (ES-G, TS-G, DHEA-G, ETIO-G and AN-G) was studied on reversed-phase columns, L-Column ODS and TSK gel ODS 120-T (250 × 4.6 mm I.D., particle size 5 μm), with aqueous methanol and aqueous acetonitrile as mobile phase. Moreover, the effect of triethylamine, acetic acid, sodium phosphate and sodium lauryl sulfate in the mobile phase on the tailing of the peak due to the reagent DMEQ-hydrazide were examined. The tailing of the peak due to the reagent was minimized by the addition of triethylamine into the mobile phase as a counter-ion against the reagent. The best separation was attained on L-Column ODS with methanol–acetonitrile–0.5% (1:1:2, v/v) triethylamine in water as the mobile phase. Fig. 2 shows a typical chromatogram obtained with the five glucuronides. All the peaks were completely separated and eluted within 40 min. The fluorescence excitation (maximum, 367 nm) and emission (maximum, 445 nm) spectra of the DMEQ derivatives of the individual glucuronides in the eluates from the column were virtually identical with those of DMEQ-hydrazide in the mobile phase. The individual glucuronides gave single peaks in the chromatograms.

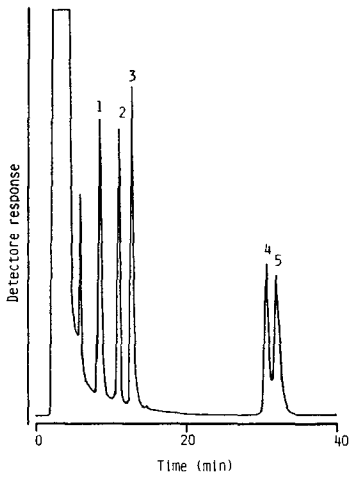

Fig. 2. Chromatogram of DMEQ derivatives of glucuronide conjugates. An aliquot (5 μl) of a standard mixture of the glucuronides (each 1.0 nmol/ml) was treated according to the described procedures. Peaks: 1 = ES-G; 2 = TS-G; 3 = DHEA-G; 4 = ETIO-G; 5 = AN-G.

3.2. Derivatization conditions

The conditions were examined using a mixture of the five glucuronide conjugates (1.0 nmol/ml each). The most intense peaks were obtained at concentrations greater than 5.0 m*M* of DMEQ-hydrazide solution for all the glucuronides; 10 m*M* was used in the derivatization of the standard glucuronides solutions. EDC and pyridine were used to facilitate the derivatization of the glucuronides with DMEQ-hydrazide. Maximum and constant peak heights could be attained at pyridine concentrations in the solution in the range of 1–5%; 4% was selected as optimum. The peak heights for the glucuronides were maximal and constant at concentrations of EDC higher than 2.0 *M*; 4.0 *M* was employed. The effect of the reaction solvents on the fluorescence derivatization was examined using methanol, ethanol, acetonitrile, N,N-dimethylformamide, dimethylsulphoxide and their mixtures. A mixture of N,N-dimethylformamide and ethanol gave the most intense and constant peak heights independent of their composition ratio; water did not inhibit the derivatization in the range 1–33% (v/v) in the derivatization solvents. On the other hand, the yields of DMEQ derivatives of the glucuronides increased in the presence of hydrochloric acid in the pyridine solution. Hydrochloric acid gave maximum and constant peak heights at concentrations of 10–30 m*M* in the pyridine solution. Thus, N,N-dimethylformamide, water and 20 m*M* hydrochloric acid ethanolic solution were used for the preparation of the DMEQ-hydrazide solution, glucuronide stock solution and pyridine solution, respectively.

The derivatization reaction of the glucuronides with DMEQ-hydrazide occurred even at 0°C; higher temperature allowed the fluorescence to develop more rapidly (Fig. 3). However, at 60°C, the peak heights decreased at long-time

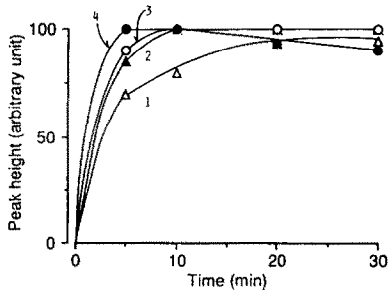

Fig. 3. Effect of reaction temperature and time on the peak height. Aliquots (5 μl) of a standard mixture of the glucuronides (each 1.0 nmol/ml) were treated according to the procedure, except for temperature. Temperature: (1) 0°C; (2) 25°C; (3) 37°C; (4) 60°C.

heating. At 37°C, the peak heights for all the glucuronides were almost maximal after heating for 10 min. Thus, heating for 20 min at 37°C was employed in the procedure. The DMEQ derivatives in the final mixture were stable for at least 24 h in daylight at room temperature.

3.3. Precision, calibration curves and detection limits

The precision was established by repeated determination using a standard mixture of the glucuronides (1.0 nmol/ml each). The relative standard deviations did not exceed 3.5% for all the glucuronides examined ($n = 10$ in each case). The relationships between the peak heights and the amounts of the individual glucuronides were linear up to at least 5.0 pmol per injection volume (10 μl); the linear correlation coefficients were 0.995 or better for all the glucuronides. The detection limits (signal-to-noise ratio = 3) for the glucuronides are 12–33 fmol for an injection volume of 10 μl (120–330 fmol per 5 μl of human urine).

3.4. Reaction of DMEQ-hydrazide with other compounds

Glucuronide conjugates of several steroids listed in Table 1 and uronic acids (glucuronic acid and galacturonic acid) reacted with DMEQ-hydrazide under the derivatization conditions recommended to give the corresponding fluorescent derivatives. The retention times and detection limits for the compounds are shown in Table 1. Other substances, such as 17 different α-amino acids, alcohols, sugars, amines, ketones, phenols and sulfhydryl compounds, gave no fluorescent derivatives under these conditions.

3.5. Application to the determination of ETIO-G and AN-G in human urine

In order to investigate the availability of the present HPLC method to biological materials, the method was applied to the determination of ETIO-G and AN-G, which are very important as a clinical indicator of adrenal functions in human urine. A typical chromatogram obtained with normal human urine is shown in Fig. 4. The peaks for the glucuronides were identified on the basis of their retention times and the fluorescence excitation and emission spectra of the eluates in comparison with the standard compounds, and also by co-chromatography of the standards and urine sample with aqueous 40–100% acetonitrile or aqueous 50–100% methanol as mobile phases.

The direct derivatization conditions for ETIO-G and AN-G in human urine were examined as to DMEQ-hydrazide, pyridine and hydrochloric

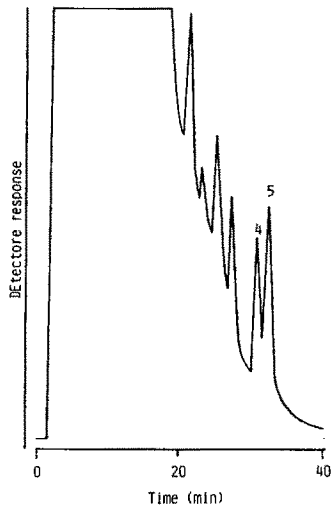

Fig. 4. Chromatogram of DMEQ derivatives of ETIO and AN in normal human urine. An aliquot (5 μl) of urine was treated as described. Peak numbers as in Fig. 2.

acid concentrations. The resulting optimal conditions were similar to those in the standard glucuronide solutions except for DMEQ-hydrazide concentration. When 50 m*M* DMEQ-hydrazide solution was used, the most effective derivatization of the glucuronides in human urine was attained; 40 m*M* or less gave low derivatization yields because of unknown urinary interferences. The recoveries ($n = 8$) of ETIO-G and AN-G (35 pmol per 5 μl each) added to a pooled normal urine were 101.3 and 104.1%, respectively.

The concentrations of ETIO-G and AN-G in urine from healthy volunteers determined by this direct derivatization method are given in Table 2. The mean values for the individual glucuronides in normal human urine were in good agreement with the published data [4].

Compounds except for ETIO-G and AN-G listed in Table 1 were eluted within 25 min under the same HPLC conditions as those described in Figs. 2 and 4. When urine, however, was treated as in the procedure, large peaks due to urinary endogenous substances other than the compounds appeared at retention times of 2–25 min on the chromatogram (Fig. 4). Therefore, the compounds could not be successfully detected in human urine by the method. Studies on an improved method for the determination of these compounds are in progress.

DMEQ-hydrazide is highly sensitive and reacts readily with glucuronide conjugates via the glucuronic acid moiety even in aqueous solution at moderate temperature to give the corresponding DMEQ derivatives. Accordingly, the HPLC method with fluorescence detection using DMEQ-hydrazide is suitable for the determination of thermally labile and polar glucuronide conjugates of biologically important substances. The method was successfully applied to the determination of ETIO-G and AN-G in human urine. The method is based on the direct derivatization of the glucuronides in urine, and does not requires conventional liquid–liquid extraction. Thus, the method is simple and rapid to perform. Therefore, the method should be useful for the biomedical investigation of glucuronide conjugated metabolites of several biologically important substances.

Table 2
Concentrations of ETIO and AN in urine from normal subjects

Age	Sex[a]	AN-G (μmol/day)	ETIO-G (μmol/day)
44	M	11.4	5.56
34	M	11.4	4.60
27	M	9.94	5.34
23	M	7.87	5.32
23	M	13.9	4.53
22	M	7.71	7.32
Mean		10.37	5.44
S.D.		2.16	0.92
23	F	6.57	3.77
23	F	7.42	3.77
22	F	4.83	2.33
21	F	1.40	1.10
21	F	9.09	3.66
21	F	7.28	4.49
Mean		6.10	1.13
S.D.		2.45	1.24

[a] M = male; F = female.

4. References

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