Journal of Chromatography, 341 (1985) 426—431
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2563

Note

3-Chloroformyl-7-methoxycoumarin as a fluorescent derivatization reagent for alcoholic compounds in liquid chromatography and its use for the assay of 17-oxosteroids in urine

CHIZUKO HAMADA and MASATAKE IWASAKI

Daiichi College of Pharmaceutical Sciences, Tamagawa, Minami-ku, Fukuoka 815 (Japan)

and

NAOTAKA KURODA and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

(First received November 2nd, 1984; revised manuscript received January 16th, 1985)

Several fluorogenic reagents have been reported for the derivatization of alcoholic compounds in high-performance liquid chromatography (HPLC); i.e. 4-dimethylamino-1-naphthoyl nitrile (DANN) [1], 2-methyl-1,1′-binaphthalene-2′-carbonyl nitrile (MBCN) [2], 1- and 9-anthroyl nitriles (1-AN and 9-AN) [3] and 4-diazomethyl-7-methoxycoumarin (DMC) [4]. Although it has been shown that 3-chloroformyl-7-methoxycoumarin (3CMC) gives fluorescent esters when refluxed with methanol and ethanol and it is readily synthesized [5], no further study of its application in liquid chromatography has been described.

We have found that MC3C also reacts sensitively with primary alcohols other than methanol and ethanol and secondary alcoholic compounds in acetone, benzene or their mixture at high temperatures to yield highly fluorescent esters, which can be separated by thin-layer chromatography (TLC) or HPLC. As an example in the practical use of the reagent, we have developed an HPLC method for the determination of 17-oxosteroids (androsterone, dehydroepiandrosterone and etiocholanolone), which have a secondary hydroxyl group in the molecule, in a small amount of human urine.

0378-4347/85/$03.30 © 1985 Elsevier Science Publishers B.V.

EXPERIMENTAL

Materials and apparatus

All chemicals were of analytical-reagent grade unless otherwise noted. Deionized and distilled water was used. Organic solvents were distilled and dried in the usual manner. 3CMC was synthesized as described by Baker and Collis [5]. TLC plates were prepared by dispersing 30 g of silica gel (Wakogel-B5, Wako, Osaka, Japan) in 60 ml of water, applying the dispersion on glass plates (20 × 20 cm) at a thickness of 0.25 mm with a TLC spreader (Yazawa Kagaku, Tokyo, Japan) and heating the plates at 120°C for 1 h for the activation. Human urine samples were obtained from healthy volunteers of the Faculty of Pharmaceutical Sciences, Kyushu University.

Uncorrected fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorimeter using 1 × 1 cm quartz cells; spectral bandwidths of 10 nm were used in both the excitation and emission sides of the monochromator. A Jasco TWINCLE chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injector valve (20-μl loop) and a Hitachi 650-10S spectrofluorimeter fitted with a 18-μl flow-cell operating at 400 nm emission and 355 nm excitation; spectral bandwidths of 10 nm were used in both the excitation and emission sides of the monochromator. A stainless-steel column (150 × 4.0 mm I.D.) was packed with TSK gel ODS-120A (particle size 5 μm; Toyo Soda Kogyo, Tokyo, Japan) using a slurry technique with chloroform as solvent.

Procedure for TLC and HPLC of the reaction mixtures of alcoholic compounds

A mixture of 50 μl each of test solution in benzene or acetone and 10 m*M* 3CMC solution in benzene was placed in a screw-capped reaction vial (3.5 ml; Gasukuro Kogyo, Tokyo, Japan), which was heated at 100°C for 20 min. A 5-μl aliquot of the reaction mixture was applied on a TLC plate and then developed with benzene—ethyl acetate (8:2) at ca. 23°C. The fluorescent band of the ester was scraped off and the ester was extracted with 3.5 ml of chloroform. The reaction mixture (20 μl) was also injected into the chromatogram after the dilution with 0.9 ml of methanol and eluted with aqueous 70% methanol containing 2% acetic acid at a flow-rate of 0.5 ml/min for the esters of alcohols, with aqueous 80% methanol containing 2% acetic acid at a flow-rate of 1.0 ml/min for the esters of 17-oxosteroids and 17-α-hydroxypregnenolone, and with a mixture of tetrahydrofuran—methanol (1:12) containing 2% acetic acid at a flow-rate of 1.0 ml/min for cholesterol and cholestanol.

Assay procedure for 17-oxosteroids in urine

To 0.5 ml of urine were added 0.2 ml of 2 *M* acetate buffer (pH 5.2) and 10 μl of a suspension of β-glucuronidase containing arylsulphatase (β-glucuronidase activity 95 400 U/ml, arylsulphatase activity 5110 U/ml; Sigma, St. Louis, U.S.A.) and the mixture was incubated at 37°C overnight to hydrolyze the conjugated forms of 17-oxosteroids [6]. To the resulting mixture, 5.0 ml of dichloromethane were added and mixed on a vortex-type mixer for 1 min. The organic layer (2.0 ml) was evaporated to dryness in vacuo below 30°C and the residue was dissolved in 0.5 ml of acetone by shaking on a

vortex-type mixer for 1 min. A 50-μl aliquot of the solution was treated as described in the procedure for HPLC. For the establishment of calibration curves, a series of 17-oxosteroid standard solutions (50—500 nmol/ml for each of the steroids, in acetone) was prepared, and the mixtures of water (0.5 ml, in place of urine sample) and standards (50 μl) were treated as described above without incubation. The peak heights in the chromatogram were used for the quantification of 17-oxosteroids.

RESULTS AND DISCUSSION

Reaction 3CMC with alcholic compounds and separation of their esters by TLC and HPLC

3CMC esters of alcoholic compounds examined (see Table I) have the fluorescence excitation and emission maxima around 355 and 400 nm, respectively, in methanol, acetone, ethyl acetate, chloroform, benzene or *n*-hexane, and the fluorescence increases in intensity with increasing polarity of the solvents.

The reaction of 3CMC with primary alcohols (see Table I) in benzene or acetone is complete within 60 min at 25°C or within 7 min at 100°C in a tightly closed reaction vial, whereas the reaction with secondary alcoholic compounds, including hydroxysteroids (see Table I), is complete within 20 min at 100°C.

Benzene and acetone as solvents for the reaction provide the most intense fluorescence for all the compounds examined; other solvents tested, acetonitrile, dioxane and tetrahydrofuran, give less intense fluorescence. Water interferes with the reaction. Acceleration of the reaction was not observed in the presence of trichloroacetic acid, acetic acid, pyridine, triethylamine, potassium carbonate or 15-crown-5 (a phase-transfer catalyst). Thus, the reaction in benzene, acetone or their mixture at 100°C for 20 min was employed in the procedure. Tertiary alcohols, such as 2-methyl-2-propanol and 2-methyl-2-butanol, did not react with the reagent.

TABLE I

R_F VALUES AND LIMITS OF DETECTION FOR 3CMC ESTERS OF ALCOHOLIC COMPOUNDS IN TLC

Compound	R_F	Detection limit (pmol)
Reaction blank	0.00	
	0.38	
Methanol	0.31	41
Ethanol	0.37	33
l-Pentanol	0.50	22
Benzyl alcohol	0.49	8
2-Propanol	0.44	82
2-Pentanol	0.52	82
Cyclohexanol	0.49	200
Etiocholanolone	0.48	2
Androsterone	0.50	2
Dehydroepiandrosterone	0.44	5
17-α-Hydroxypregnenolone	0.22	9
Cholesterol	0.61	15
Cholestanol	0.62	5

3CMC is stable in benzene for ten days or more at 25°C and in acetone for 8 h; a benzene solution of 3CMC was used in the recommended procedure. The reaction mixture of the hydroxyl compounds diluted ten times with methanol can be stored at room temperature (ca. 23°C) for at least three days in daylight.

3CMC esters of the compounds tested can be separated by TLC on silica gel plates and show single fluorescent bands (Table I). The limits of detection are at the picomole level, and in particular the sensitivity for hydroxysteroids is high (Table I). The 3CMC esters extracted from the bands on the TLC plate with chloroform are stable for ca. three days when dissolved in methanol, acetone or benzene after the removal of the extraction solvent. These observations indicate that 3CMC is usable as a fluorescent derivatization reagent for alcoholic compounds in liquid chromatography.

The esters of alcohols and hydroxysteroids, except those of cholesterol and cholestanol, are separated within 20 min by reversed-phase HPLC on a TSK gel ODS-120A column using aqueous 70% and 80% methanol containing a small amount of acetic acid as mobile phases, respectively (Fig. 1A and B). The peaks for benzyl alcohol and cyclohexanol cannot be separated under the HPLC conditions. The peaks of methanol, ethanol and 2-propanol overlap that of the reagent blank. The esters of cholesterol and cholestanol are eluted within 12 min with methanol containing small amounts of tetrahydrofuran and acetic acid (Fig. 1C) though they are retained on the column when aqueous methanol is used as mobile phase.

The calibration curves for the alcoholic compounds (see Fig. 1) are linear up to at least 2 nmol/ml. The limits of detection for alcohols and hydroxysteroids are 0.12—0.50 and 0.11—0.23 pmol in a 20-μl injection volume, respectively, at

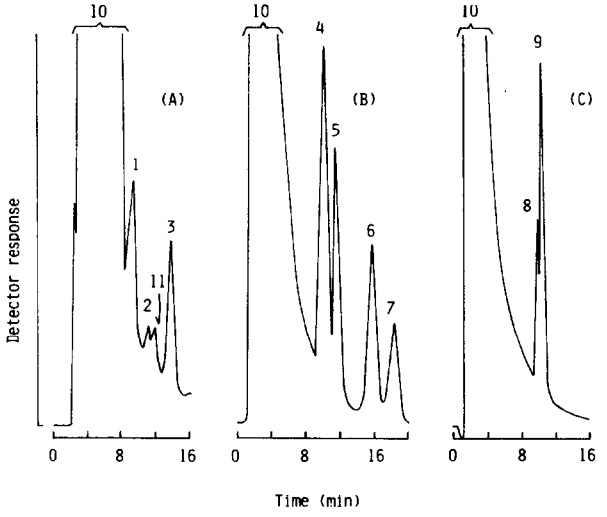

Fig. 1. Chromatograms of 3CMC esters of alcohols (A) and hydroxysteroids (B and C). Portions (50 μl) of a mixture of alcoholic compounds (alcohols, 50 μ*M* each; hydroxysteroids, 10 μ*M* each; in benzene) were treated according to the procedure for HPLC. Peaks: 1 = benzyl alcohol and cyclohexanol; 2 = 2-pentanol; 3 = 1-pentanol; 4 = etiocholanolone; 5 = androsterone; 6 = dehydroepiandrosterone; 7 = 17-α-hydroxypregnenolone; 8 = cholesterol; 9 = cholestanol; 10 = the components of the reagent blank; 11 = one of the components of the reagent blank.

a signal-to-noise ratio of 2. This sensitivity is almost the same as those in 1-AN and 9-AN and at least there times higher than those in DANN and MBCN.

3CMC also reacts with aliphatic primary and secondary amines such as 1-propylamine, 3-methyl-2-propylamine, benzylamine, 2-furfurylamine, di-*n*-butylamine, piperidine, spermine and histamine under the reaction conditions described. These aliphatic amines, however, do not interfere with the determination of alcoholic compounds by HPLC because their 3CMC derivatives can be eluted earlier than the 3CMC esters (within 4 min) under the HPLC conditions. 3CMC does not react with aromatic amines such as aniline and α-naphthylamine and catecholamines (dopamine, epinephrine and norepinephrine). No fluorescent derivative is provided from the reaction of 3CMC with phenolic compounds such as phenol and salicylic acid, but DANN, 1-AN and 9-AN give fluorescent products from phenolic compounds. DMC reacts with carboxylic compounds to form fluorescent derivatives. These observations indicate that 3CMC is more selective than the other reagents for alcoholic compounds.

Assay for 17-oxosteroids in human urine

We developed a sensitive HPLC method for the assay of totals of individual 17-oxosteroids in urine on the above-mentioned basis. The conjugated 17-oxosteroids are hydrolyzed by β-glucuronidase- and arylsulphatase-mediated reactions in the usual manner [6], and the resulting free 17-oxosteroids are extracted with dichloromethane. The steroids remaining after removal of the solvent from the extract are dissolved in acetone, and the acetone solution is subjected to derivatization with 3CMC followed by HPLC.

Fig. 2 shows a typical chromatogram obtained from the urine of a healthy man according to the assay procedure. Linear relationships were observed between the peak heights and the concentrations of androsterone, dehydroepiandrosterone and etiocholanolone up to at least 25 nmol/ml.

The recoveries of androsterone, dehydroepiandrosterone and etiocholanolone

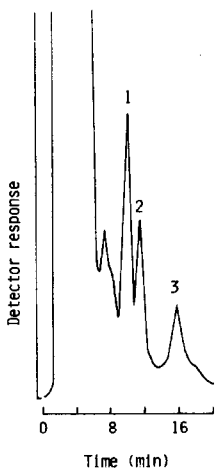

Fig. 2. Chromatogram from normal urine. Peaks (concentration in μg/ml): 1 = etiocholanolone (5.1); 2 = androsterone (2.8); 3 = dehydroepiandrosterone (3.0).

(10 nmol/ml of each) from urine were 95 ± 5.0, 92 ± 2.8 and 90 ± 6.2% (mean ± S.D., $n = 10$ for each), respectively.

The limits of detection for androsterone, dehydroepiandrosterone and etiocholanolone were 0.17, 0.28 and 0.15 pmol in a 20-μl injection volume (which correspond to 98, 162 and 87 ng/ml in urine), respectively, at a signal-to-noise ratio of 2. The sensitivity seems to be at least five times higher than that of the HPLC method using Dns hydrazine [7]. The precision was established by repeated assays ($n = 10$) using normal urine containing androsterone, dehydroepiandrosterone and etiocholanolone at 1.8, 1.8 and 2.2 μg/ml, respectively. The coefficients of variation were 2.8, 3.9 and 5.5%, respectively. The amounts (mg, mean ± S.D.) of androsterone, dehydroepiandrosterone and etiocholanolone in the 24-h urine of healthy persons assayed by this method were 2.6 ± 0.8, 2.2 ± 0.9 and 4.9 ± 0.7 in men (22—26 years, $n = 5$), and 1.2 ± 0.7, 0.9 ± 0.8 and 3.4 ± 0.8 in women (22—23 years, $n = 5$), respectively. The values of individual 17-oxosteroids are in good agreement with the published data [7].

This study shows that 3CMC should be useful as a fluorescent derivatization reagent in liquid chromatography of alcoholic compounds.

REFERENCES

1 J. Goto, S. Komatsu, N. Goto and T. Nambara, Chem. Pharm. Bull., 29 (1981) 899.
2 J. Goto, N. Goto and T. Nambara, Chem. Pharm. Bull., 30 (1982) 4597.
3 J. Goto, N. Goto, F. Shamsa, M. Saito, S. Komatsu, K. Suzaki and T. Nambara, Anal. Chim. Acta, 147 (1983) 397.
4 A. Takadate, T. Tahara, H. Fujino and S. Goya, Chem. Pharm. Bull., 30 (1982) 4120.
5 W. Baker and C.B. Collis, J. Chem. Soc., (1949) S12.
6 H.Ch. Curtius and M. Roth (Editors), Clinical Biochemistry — Principles and Methods, Walter de Gruyter, Berlin, New York, 1978, p. 700.
7 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 226 (1981) 1.