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Short Communication

Application of a metal capillary column in gas chromatographic determination of catechol-O-methyltransferase activity

SEISHU KOH, KOJI URAYAMA and SATOSHI KAWAI*
Gifu Pharmaceutical University, Mitahora, Gifu 502 (Japan)
and
YUZI TAKAYAMA
Department of Materials Science, Toyohashi University of Technology, Tempaku, Toyohashi 440 (Japan)
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ABSTRACT

The utility of a deactivated metal capillary column, Rascot, in the measurement of an enzymatic reaction, in this case measurement of rat catechol-O-methyltransferase activity, was examined. 3,4-Dihydroxybenzaldehyde, 3,4-dihydroxybenzylalcohol and 3,4-dihydroxybenzoic acid were used as substrates and the *m*- and *p*-O-methylated products were separated by using Rascot after derivatization. The peaks on the chromatograms were symmetrical. The data obtained were compared with those reported in previously published papers. Good agreement with previous results proved that Rascot is able to withstand practical use in biological materials.

INTRODUCTION

Capillary gas chromatography (GC) has a long history. The first metal capillary columns were very far from being of practical use, because of strong adsorption of solutes, especially polar ones.

Recently, we have published some papers on the use of a deactivated metal capillary column, Rascot, which has been shown to be of much practical use [1–5].

GC [6,7] and high-performance liquid chromatographic [8–12] techniques have been developed as reliable procedures for the measurement of catechol-O-methyltransferase (COMT) activity. The present paper describes the assay of COMT activity using the following three compounds as substrates: 3,4-dihydroxybenzaldehyde (DHBAd), 3,4-dihydroxybenzylalcohol (DHBAl) and 3,4-dihydroxybenzoic acid (DHBAc). The utility of Rascot was examined by separating the *m*- and *p*-O-methylated products.

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EXPERIMENTAL

Materials

S-Adenosyl-L-methionine (SAM) hydrogen sulphate was purchased from Boehringer (Mannheim, Germany). Vanillyl alcohol, vanillin, isovanillin, isovanillic acid, *p*-diiodobenzene and homovanillic acid were obtained from Tokyo Kasei Kogyo (Tokyo, Japan), vanillic acid from Sigma (St. Louis, MO, USA) and isovanillyl alcohol from Aldrich (Milwaukee, WI, USA). DHBAd and DHBAc were obtained from Wako Junyaku Kogyo (Osaka, Japan) and DHBAl was synthesized. All other reagents were of analytical grade and purchased from commercial sources.

Preparation of DHBAl

DHBAl was synthesized by the reduction of DHBAd. A 0.3-g sample of palladium–carbon was put in a flask, and 30 ml of methanol were slowly added to it. To the solution mixture were added 0.3 g of DHBAd, which was reduced on the palladium–carbon with hydrogen. The reaction mixture was filtered and the filtrate was evaporated to dryness. The residue was recrystallized from 2 ml of benzene containing three drops of ethyl acetate. m.p. 134–135°C (reported 136°C). Elemental analysis for $C_7H_3O_3$: calculated, C 59.77%, H 5.79%; found, C 59.64%, H 5.77%.

Enzyme preparation

Adult Wistar rats (150–200 g) of either sex were decapitated and their livers were homogenized in four volumes of 50 m*M* phosphate buffer (pH 7.5). The homogenate was centrifuged at 100 000 *g* for 30 min at 4°C. The supernatant solution was used as a crude source of COMT to study the ratio of *m*-/*p*-methylation. Protein determinations were made by the method of Lowry *et al.* [13] with bovine serum albumin as a standard.

Apparatus and GC conditions

A gas chromatograph (Type 103C, Okura Riken) was furnished with a flame ionization detector and an on-column processor (Nippon Chromato, Tokyo, Japan; an instrument having an injector and a precolumn which is capable of rapid heating independently from the GC column oven) [1,14]. A precolumn, RAS 80 (an uncoated deactivated metal tube, 100 cm × 0.8 mm I.D.), was connected to an analytical capillary column, Rascot OV-1 (a deactivated metal column coated with OV-1, 25 m × 0.25 mm I.D., film thickness 0.4 μm, Nippon Chromato), with a metal connector.

A 5-μl aliquot of the sample solution was taken in a microsyringe with a long needle (15 cm × 0.5 mm O.D.). After the sample solution was slowly introduced into the precolumn, which was kept at room temperature, the precolumn was rapidly heated to about 150°C by a flash heater [1,14]. Next, the column oven temperature was raised from 60°C to 150°C at a rate of 4°C/min.

Assay of COMT

Procedure 1 (substrate DHBAd). The standard incubation mixture consisted of following components in a total volume of 1.0 ml in 50 m*M* phosphate buffer (pH 7.2): 2 m*M* substrate, 1 m*M* SAM, 5 m*M* magnesium chloride and 2 mg of enzyme protein. The mixture was incubated for 1 h. Incubation was stopped by adding 0.1 ml

of 1 *M* hydrochloric acid. The incubation mixture was extracted with 4 ml of chloroform containing *p*-diiodobenzene (internal standard, I.S.) under saturated sodium chloride. The extract was evaporated using a rotary evaporator. Three drops of ethyl acetate and 0.2 ml of trifluoroacetic anhydride (TFAA) were added to the residue and the derivatization was performed in a water bath at 60° for 15 min. A 5-μl aliquot of the mixture was determined by GC. Blanks were prepared by omitting SAM or the substrate.

Procedure 2 (substrate DHBAl). The standard incubation mixture consisted of the same components except in phosphate buffer (pH 7.6) and 1 m*M* substrate, and incubation and derivatization were performed in the same manner as in procedure 1.

Procedure 3 (substrate DHBAc). The standard incubation mixture and incubation conditions were the same as in procedure 1. Incubation was stopped by adding 0.1 ml of 1 *M* hydrochloric acid. The incubation mixture was extracted with 4 ml of chloroform containing homovanillic acid (I.S.) under saturated sodium chloride. The extract was evaporated using a rotary evaporator. To the residue, 0.1 ml of ethyl acetate and 0.2 ml of diazomethane in diethyl ether were added and the solution was allowed to stand in iced water for 10 min. After evaporating again, 0.1 ml of ethyl acetate and 0.2 ml of TFAA were added to the residue and the solution was allowed to stand at room temperature for 30 min. A 5-μl aliquot of the solution was injected into the gas chromatograph.

RESULTS AND DISCUSSION

At present, fused-silica columns are exclusively used for capillary GC owing to their inert surface and flexibility. Metal capillary columns seem to have been abandoned mainly because of the strong adsorption of solutes on the first metal capillaries. However, the problem of the brittleness of fused-silica capillaries still awaits solution. Although they are flexible, we often find that breakage occurs during handling. After a long time of use, especially at high temperature, the material becomes brittle, because the outer protective polyimide coating is easily damaged and cannot withstand high temperature. Recently, an excellent technique of deactivating the inner surface of metal capillaries was developed by Takayama, and a deactivated metal capillary column named Rascot has been put on the market by Nippon Chromato. We previously reported an excellent separation of a polarity mixture containing 2,3-butanediol, *n*-butyric acid, 2,6-xylenol, 2,6-xylidine, 1-decanol and some alkanes using this Rascot [2] and confirmed its inertness. In the present study we wanted to demonstrate the usefulness of Rascot in the determination of compounds in biological samples as well as pure standard compounds, and the measurement of rat COMT activity was selected as a typical example. In order to decrease the polarity and increase the volatility of the O-methylated products obtained from incubation with the substrate and COMT according to the procedures described, the derivatizations were performed prior to injection into the gas chromatograph. Three typical separations of the derivatives are illustrated in Fig. 1. Rascot was chemically and physically inert. The peaks on the chromatograms were symmetrical, without tailing or leading, and there were no interfering components. In the blanks no O-methylated products were formed.

The metal capillary column was mechanically though and very easy to handle

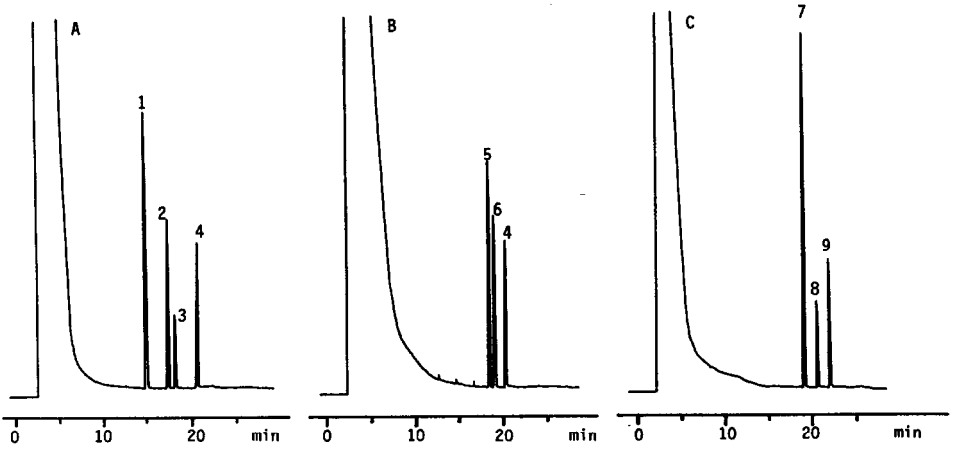

Fig. 1. Typical separation of O-methylated products obtained from incubation with three different substrates and COMT through their derivatives according to the procedures described. Substrates: A, DHBAd; B, DHBAl; C, DHBAc. Peaks: 1 = DHBAd; 2 = vanillin-TFA; 3 = isovanillin-TFA; 4 = *p*-diiodobenzene (I.S.); 5 = vanillyl alcohol-TFA; 6 = isovanillyl alcohol-TFA; 7 = vanillic acid-CH_3-TFA; 8 = isovanillic acid-CH_3-TFA; 9 = homovanillic acid-CH_3-TFA (I.S.): TFA = trifluoroacetate; CH_3-TFA = methyl ester-trifluoroacetate. For GC conditions, see Experimental section.

without breakage in small oven. In addition, the durability of its inertness was superior to that of fused-silica capillaries and it had the advantage of being usable for a very long time. Furthermore, a special sampling technique, "on-column processor", has also been developed by Takayama and Sumiya [14]. When a large sample volume is injected, this technique makes the injection easy and prevents degradation of the stationary phase. Use of the uncoated precolumn results in sharp peaks because of the solvent effect on the head of the analytical capillaries or the cold-trapping effect after the solvent has gone [15].

Chloroform was a suitable extraction solvent against substrates and for products. The recoveries from solutions containing of 100 nmol/ml of each compound were 93.2% for vanillin, 98.0% for isovanillin, 95.4% for vanillyl alcohol, 76.2% for isovanillyl alcohol, 100.0% for vanillic acid and 100.4% for isovanillic acid.

Methylation of vanillic acid and isovanillic acid was carried out in iced water for 10 min, where O-methylation of the phenolic hydroxyl group was not observed. Trifluoroacetylation of the products (vanillin, isovanillin, vanillyl alcohol and isovanillyl alcohol) was achieved by heating at 60°C for 15 min, while the derivatization of methyl esters of vanillic acid and isovanillic acid with TFAA proceeded completely at room temperature for 30 min.

The calibration graphs which were prepared from ratios of peak height of the I.S. were linear in the range from 10 to 150 nmol/ml vanillin (the correlation coefficient was 0.999), isovanillin (0.999), vanillyl alcohol (0.994), isovanillyl alcohol (0.992), vanillic acid (0.998) and isovanillic acid (0.998) in their reaction mixtures, and the detection limits at a signal-to-noise ratio of 2 were about 4 nmol/ml. The mean relative standard deviations for replicate assays (n = 5) using an identical parent solution spiked with 50 nmol/ml of each component were from 2.8% to 5.4%.

TABLE I

KINETIC PARAMETERS OBTAINED WITH THE THREE SUBSTRATES

Substrate	*Meta/para* ratio at pH 7.2	K_m (m*M*)	V_{max} (nmol/mg protein/min)
DHBAd	2.38	*Meta* 0.21	0.74
		Para 0.30	0.37
DHBAl	1.29	*Meta* 0.12	1.00
		Para 0.09	0.74
DHBAc	4.50	*Meta* 0.57	8.34
		Para 0.40	1.39

The kinetic parameters of rat liver COMT were measured by this GC method and the results are shown in Table I.

It is now clear that the *in vitro meta/para* ratio depends on a variety of factors, including pH in the incubation solution and the nature of substituent on the catechol ring [6,8].

In order to confirm the influence of the side-chain on the *meta/para* ratio, we used the three different substrates, DHBAd, DHBAl and DHBAc, which have characteristic properties on their side group and their *m*- and *p*-O-methylated products are easily be derivatized for GC.

The apparent Michaelis–Menten coefficients (K_M) were approximately $2.1 \cdot 10^{-4}$ *M* for vanilline, $3.0 \cdot 10^{-4}$ *M* for isovanillie, $1.2 \cdot 10^{-4}$ *M* for vanillyl alcohol, $8.6 \cdot 10^{-5}$ *M* for isovanillyl alcohol, $5.7 \cdot 10^{-4}$ *M* for vanillic acid and $4.0 \cdot 10^{-4}$ *M* for isovanillic acid.

The ratio of *meta* in *para* of the methylated products using the three different substrates was also calculated at pH 7.2, and the results are shown with the K_M values and V_{max} values in Table I. The K_M values for DHBAl were the lowest of the three, while those for DHBAc were higher and their V_{max} values were also higher.

Several studies dealing with the ratio of *m*/*p*O-methylation from rat liver COMT have been presented [6,8]. Creveling *et al.* [6] have reported that *p*-methylation is not favored by the presence of either a cationic or an anionic substituent, while neutral substituents favor a lower *meta/para* ratio. The *meta/para* ratio of O-methylatan of DHBAc was 4.5 at pH 7.2 and 6.0 at pH 8, which was in good agreement with the value (5.5) obtained at pH 8.0 by Creveling *et al.* [6] and was similar to that (4.6) obtained at pH 7.9 by Pennings and Van Kempen [8], and the ratios for DHBAd (2.38) and DHBAl (1.29) were lower and were also close to those (2.1 and 2.0, respectively) reported by Creveling *et al.* [6]. The good agreement between these results proves that Rascot is able to stand practical use in biological materials.

As far as we are aware, there have been only two papers [6,7] on GC for the assay of COMT activity. GC seems to be one of the most useful methods with regard to determination of *m* and *p* isomers. We have also proposed a simple and specific GC method for the measurement of rat COMT activity. A metal capillary column has been applied for the first time to the study of an enzymatic reaction.

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