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Synthesis and Use of Deuterated 3,4-Dihydroxyphenylglycolaldehyde as an Internal Standard for Determination of Dopegal in Brain Tissue by Gas Chromatography–Mass Spectrometry

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Trideuterated 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL-d_3) was synthesized from tetradeuterated catechol. After derivatization with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, the identity of DOPEGAL-d_3 was confirmed by comparison with unlabeled DOPEGAL by gas chromatography–mass spectrometry (GC–MS); they have the same pattern of mass fragmentation. The deuterated DOPEGAL was used as an internal standard for the determination of DOPEGAL in human locus ceruleus by GC–MS in the selected-ion-monitoring mode.

INTRODUCTION

3-4-Dihydroxyphenylglycolaldehyde (DOPEGAL) is formed by oxidative deamination of noradrenaline (NA) by monamine oxidase (MAO-A) (*1*, *2*). In tissues this aldehyde is either enzymatically reduced to 3,4-dihydroxyphenylglycol (DOPEG) or oxidized to 3,4-dihydroxymandelic acid (*1*, *3*). Although estimates of this compound in tissues have been made (*3–6*), it has not been previously measured in human brain using gas chromatography–mass spectrometry (GC–MS). This has been due to lack of deuterated DOPEGAL internal standard.

We have reported both biological (*6*) and chemical (*7*) synthesis of DOPEGAL. This DOPEGAL standard enabled the development of a method using high-performance liquid chromatography with electrochemical detection (HPLC–EC) to measure tissue levels of DOPEGAL (*6*). However, GC–MS is a more reliable quantitative method of measuring extremely low levels of bioactive compounds in tissue. Capillary gas chromatography with selected-ion-monitoring (SIM) mass spectrometry affords a combination of great selectivity and sensitivity. The high accuracy can be obtained with the stable isotope dilution method. Using deuterated analogues as the corresponding internal standard, SIM provides an ideal basis for specific quantitative analysis by GC–MS.

We present here the synthesis and use of deuterated DOPEGAL (**1**) as an

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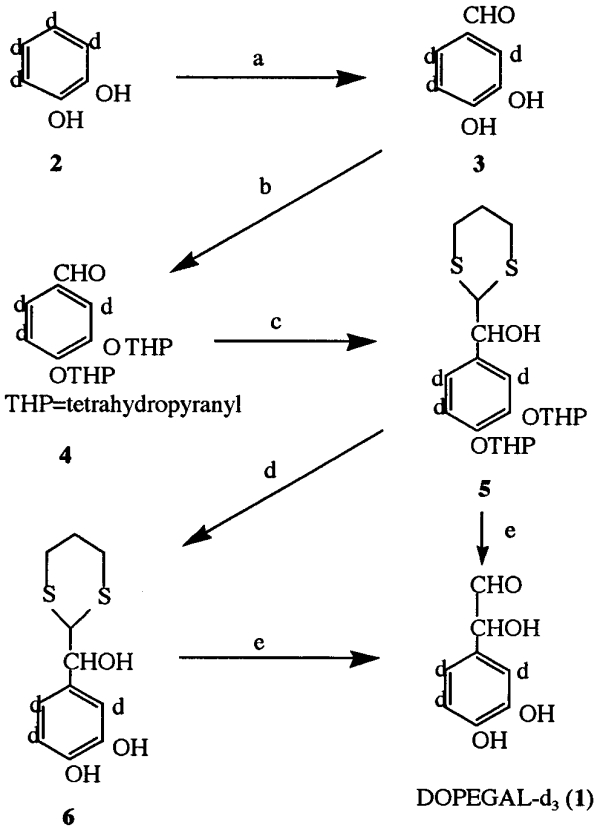

SCHEME 1. (a) *N*-Methylformanilide, $POCl_3$; (b) dihydropyran, pyridinum *p*-toluenesulfonate; (c) 2-lithio-1,3-dithiane; (d) pyridinum *p*-toluenesulfonate; and (e) $HgCl_2$, HgO, acetonitrile.

internal standard to determine the concentration of DOPEGAL in tissue samples by GC–MS–SIM. We apply this method to the measurement of DOPEGAL in human brain tissue from the locus ceruleus (LC).

The synthesis of deuterated DOPEGAL (DOPEGAL-d_3) (**1**) was performed as illustrated in Scheme 1. The deuterated catechol-d_4 (**2**) used as the starting material was prepared from catechol and deuterated water according to the procedure described by Clemo and Swan (*9*). The deuterated 3,4-dihydroxybenzaldehyde-d_3 (**3**) was prepared from **2** by utilizing *N*-methylformanilide–$POCl_3$. The synthetic route from deuterated **3** to the desired compound (**1**) was based on that of unlabeled DOPEGAL which we published (*7*). However, we optimized and modified all of the reaction conditions and some of the reaction agents were changed, such as using pyridinum *p*-toluenesulfonate instead of *p*-toluenesulfonic acid, and butyl lithium instead of lithium diisopropylamide. The last two reactions, removal of the protective group, tetrahydropyranyl, and unmasking of the desired carbonyl function, could be combined into one step by using mercury chloride and mercuric

oxide. Therefore, the purity of products was much better and they were easy to purify.

EXPERIMENTAL

Chemicals and Materials

DOPEGAL was prepared according to our previous paper (*7*). All other chemicals were purchased either from Aldrich (Milwaukee, WI) or from Sigma (St. Louis, MO). The brain tissue was obtained from the Saint Louis University Alzheimer Brain Bank. Melting points were determined on a Fisher–Johns melting point apparatus and uncorrected. IR spectra were recorded on a Perkin–Elmer 1320 spectrophotometer. Molecular masses were determined via a VG 70-250 SEQ hybrid-tandem spectrometer. All HPLC analyses were done using a Waters 6000A multisolvent delivery system with an Applied Biosystems Model 783 programmable detector. Column chromatography was performed on silica gel 60 (70–230 mesh) and TLC analysis on silica gel 60 F254 precoated plates (layer thickness 0.2 mm). Compounds for which only mass spectral data are given have purities >98% as measured by TLC or HPLC.

Synthesis of DOPEGAL-d_3 (**1**)

*3,4-Dihydroxybenzaldehyde-d*3 (**3**). A solution of catechol-d_4 (**2**) (11.4 g, 0.1 mol) in a mixture of *N*-methylformanilide (12.4 ml, 0.11 mol) and benzene (60 ml) was treated with phosphorus oxychloride (10 ml, 0.11 mol) at 10–15°C. The reaction mixture was stirred at room temperature overnight and then heated to 65–70°C for 2 h. The supernatant was poured out, and the residue was mixed with ice water, neutralized with 40% NaOH, and extracted with ethyl acetate. The extract was washed with saline, dried over anhydrous Na_2SO_4, and concentrated. The crude compound was purified by chromatography over silica gel using 2% CH_3OH in CH_2Cl_2 as eluting solvent. All the fractions corresponding to product were combined and cooled to 0°C. The separated solid was collected; yield 4.2 g (30%); mp 151–153°C. MS calcd for di-TMS derivative ($C_{13}H_{19}D_3O_3Si_2$) of 3,4-dihydroxybenzaldehyde-d_3: 285.00. Found: 285 (M^+).

3,4-Ditetrahydropyranoxybenzaldehyde-d_3 (**4**). Pyridinum *p*-toluenesulfonate (500 mg) was added to a solution of 3,4-dihydroxybenzaldehyde-d_3 (7.05 g, 0.05 mol) and dihydro-2*H*-pyran (11 ml, 0.12 mol) in CH_2Cl_2 (20 ml). The reaction mixture was stirred for 5 h at room temperature, then washed with 10% $NaHCO_3$ and saline and dried over anhydrous Na_2SO_4. Removal of the solvent gave the crude compound which was purified by chromatography on silica gel column eluting with 30% CH_2Cl_2 in hexane. The desired fractions were pooled and concentrated to afford 13 g (84%): mp 72–74°C. MS $(FAB)^+$ calcd for ($C_{17}H_{19}D_3O_5$): 309.00. Found: 310 (MH^+).

2-(3,4-Ditetrahydropyranoxy-α-hydroxybenzyl)-1,3-dithiane-d_3 (**5**). A solution of 1,3-dithiane (1.19 g, 9.9 m*M*) in 40 ml of THF was treated with 3.96 ml of 2.5 *M* butyl lithium at −60°C under nitrogen. The reaction mixture was stirred at −40°C

for 2 h and then cooled to −70°C. A solution of **4** (3.06 g, 9.9 mM) in THF (10 ml) was added to the above solution of 2-litho-1,3-dithiane at −70°C. After that, the reaction mixture was stirred at −60°C for 2.5 h and then allowed to come to room temperature overnight. Removal of the THF at room temperature gave a residue which was treated with ice water and extracted with CH_2Cl_2. The extract was washed with water, dried over anhydrous Na_2SO_4, and concentrated. The crude compound was purified by chromatography over silica gel using 30% hexane in CH_2Cl_2 as eluting solvent. All the fractions corresponding to the product were pooled and concentrated: yield 2.5 g (58.8%); IR (Nujol) 3450 (OH) cm^{-1}. MS (FAB^+) calcd for ($C_{21}H_{27}D_3S_2O_5$): 429.00. Found: 430 (MH^+).

2-(3,4-Dihydroxy-α-hydroxybenzyl)-1,3-dithiane-d_3 (**6**). Pyridinium *p*-toluenesulfonate (140 mg) was added to a solution of **5** (2.4 g, 5.6 mM) in 20 ml of 70% CH_3OH. The reaction mixture was stirred at room temperature for 5 h and extracted with ethyl acetate. The extract was washed with 10% $NaHCO_3$ and water and dried over anhydrous Na_2SO_4. Removal of solvent gave the crude compound which was recrystallized with alcohol to afford 1.29 g (88%); mp 99–100°C. MS (FAB^-) calcd for ($C_{11}H_8D_3S_2O_3$): 261.00. Found: 260 $(M-H)^+$.

DOPEGAL-d_3. Method A: To a solution of **5** (690 mg, 1.62 mM) in 70 ml of 80% acetronitrile was added mercury chloride (1.4 g) and mercuric oxide (1.5 g). The reaction mixture was refluxed for 3 h and filtered. The acetonitrile solution was concentrated and extracted with ethyl acetate. Removal of the solvent gave the crude product which was purified by chromatography over silica gel eluting with 1% CH_3OH in CH_2Cl_2. The desired fractions were pooled and concentrated to afford 174 mg (62.8%); mp 105–107°C; NMR (DMSO-d_6) δ 9.50 (d, 1H, CH). MS (FAB+) calcd for ($C_8H_5D_3O_4$): 171.00. Found: 172 (MH+).

Method B: Mercury chloride (906 mg) and mercuric oxide (718 mg) were added to a solution of **6** (321 mg, 0.75 mM) in 35 ml of 80% acetonitrile. The reaction mixture was refluxed for 3 h and extracted with ethyl acetate. The extract was washed with saline, dried over anhydrous Na_2SO_4, and concentrated. The crude compound was purified by chromatography over silica gel eluting with 1% CH_3OH in CH_2Cl_2. The desired fractions were pooled and concentrated to afford 82 mg (64%); mp 105–107°C [Lit. 105–107°C (*7*)]. MS (FAB^+) calcd for ($C_8H_5D_3O_4$): 171.00. Found: 172 (MH^+).

Derivatization of DOPEGAL and Calibration Graphs

For direct analysis of standards, the pure compounds were dissolved in acetonitrile and derivatized under nitrogen with *N*-methy-*N*-trimethylsilyltrifluoroacetamide (MSTFA) at 70°C for 1 h. A standard curve was generated by adding various amounts of unlabeled DOPEGAL to a fixed concentration of deuterated DOPEGAL. After derivatization, 1 μl of the mixture of DOPEGAL and deuterated DOPEGAL was injected into the GC–MS. Calculations were made on the basis of the DOPEGAL/DOPEGAL-d_3 peak-area ratios using the ions at *m/z* 355 and 358, respectively. The regression equation is $y = 1.057\chi + 0.521$ and the correlation coefficient (r^2) is 0.9846.

Gas Chromatography–Mass Spectrometry

A Hewlett–Packard GC5890 Series II gas chromatography coupled with a HP 5971 mass selective detector was used. The gas chromatograph was equipped with Alltech Econo Cap SE-54 capillary column (30-m × 0.25-mm i.d. × 0.25-μm film thickness). The injector temperature was 250°C. Helium was used as carrier gas (40 kPa). The initial column temperature of 70°C was held for 2 min and then increased at 20°C/min to a final temperature of 280°C. Data processing was performed using a Hewlett–Packard G1034C MS-ChemStation with Microsoft Windows Version 3.1 software. Data were stored onto a Vectra QS/20 computer. The mass spectrometer was operated at an electron energy of 70 eV and an EMV 2980. For the identification of trimethylsilyl derivatives of DOPEGAL spectra were taken in the mass range 50–500 with a scan speed of 1.5 decade/s.

Tissue Extraction and Derivatization

The LC from a 77-year-old man was dissected at autopsy after a postmortem interval (PMI) of 3.8 h. The brain tissue was homogenized in 20 volumes of acetone containing DOPEGAL-d_3 as an internal standard and kept in an ice bath for 15 min to allow protein precipitation. The homogenate was centrifuged (11,000*g*, 4°C, 15 min). The supernatant acetone solution was passed through a 0.2-μm filter into a reaction vial and evaporated under a nitrogen stream at 70°C. When the volume reached 50% of the starting volume, acetonitrile was added and concentration was continued. Finally, methylene chloride was added to remove all traces of water as an azeotropic mixture under a nitrogen stream at 70°C. MSTFA (25 μl) was added to the residue. Derivatization proceeded under nitrogen at 70°C for 1 h. Protein concentration was determined on an aliquot of tissue by the method of Lowry (*8*).

RESULTS

GC–MS Analysis

The mass spectrum of the trimethylsilyl (tri-TMS) derivative of unlabeled DOPEGAL, shown in Fig. 2, shows the molecular ion at *m/z* 384 $(C_{17}H_{32}O_4Si_3)^+$. Loss of a methyl radical produces the ion at *m/z* 369. It is generally accepted that the $(M\text{-}15)^+$ ion observed in TMS derivatives is formed by loss of a methyl group from the TMS moiety. Loss of an aldehyde radical (M-CHO) produces the ion at *m/z* 355 $(C_{16}H_{31}O_3Si_3)^+$. It is the strongest ion of DOPEGAL and a characteristic ion for DOPEGAL and we selected it as a monitor for determination of DOPEGAL. Loss of a hydrogen and TMS radical from *m/z* 355 (M-CHO-TMSH) produces the ion 281 $(C_{13}H_{21}O_3Si_2)^+$. The ion at *m/z* 147 $(TMS\text{-}O{=}Si(CH_3)_2)^+$ is observed as expected for a tri-TMS derivative. The peak at *m/z* 267 arises from *m/z* 355 by the loss of $TMSCH_3$. The route of formation of the ion at *m/z* 193 can be explained by the loss of a methyl, an aldehyde group, and 147 (M-15-29-147). The results of

TABLE 1
Mass Measurement of Tri-TMS Derivative of DOPEGAL

Formula	Calculated	Found	Ion assignments
$C_{17}H_{32}O_4Si_3$	384.00	384	(M)+
$C_{16}H_{29}O_4Si_3$	369.00	369	(M−CH_3)+
$C_{16}H_{31}O_3Si_3$	355.00	355	(M−CHO)+
$C_{14}H_{23}O_3Si_2$	295.00	295	(M−TMSO)+
$C_{13}H_{21}O_3Si_2$	281.00	281	(M-CHO-TMSH)+
$C_{12}H_{19}O_3Si_2$	267.00	267	(M-CHO-TMSCH_3)+
$C_{10}H_{13}O_2Si$	193.00	193	(M-CHO-TMSOTMS)+

measurement on the molecular ion and major fragments are given in Table 1. The mass spectrum of the tri-TMS derivative of DOPEGAL-d_3 shown in Fig. 1 shows the same pattern observed from DOPEGAL.

Analysis of DOPEGAL in Brain Tissue

The method described above was used to measure the amount of DOPEGAL in human brain tissue. Treatment of the tissue and sample preparation was as previously described, except as noted here. The MSD control program was configured for SIM during the interval of 3–13.5 min postinjection. Under the GC condition applied, the retention time for TMS derivatives of DOPEGAL was 11.3 min, for norepinephrine, 7.5 min, for epinephrine, 7.8 min. for 3,4-dihydroxyphenylglycol-TMS, 13 min, and for dihydroxy mandelic acid-TMS, 13.3 min. Therefore, interfer-

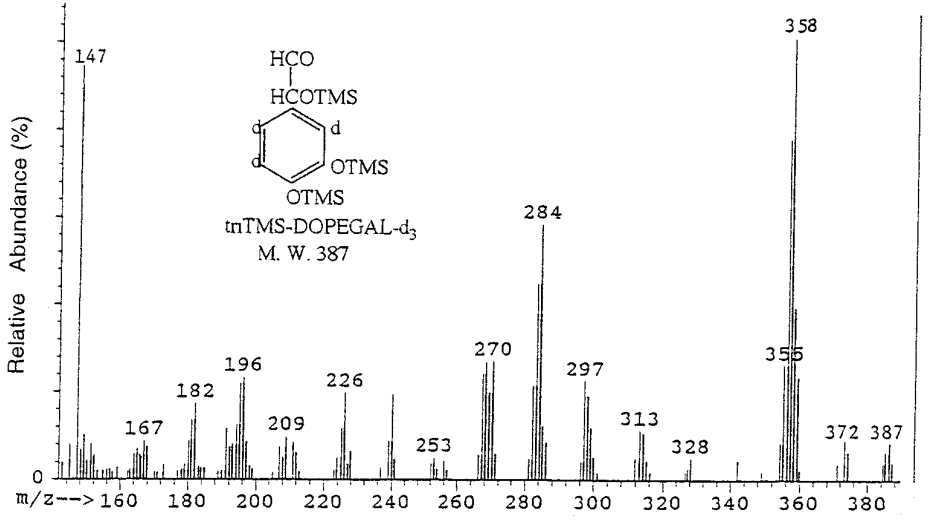

FIG. 1. Mass spectrum of tri-TMS derivative of deuterated DOPEGAL.

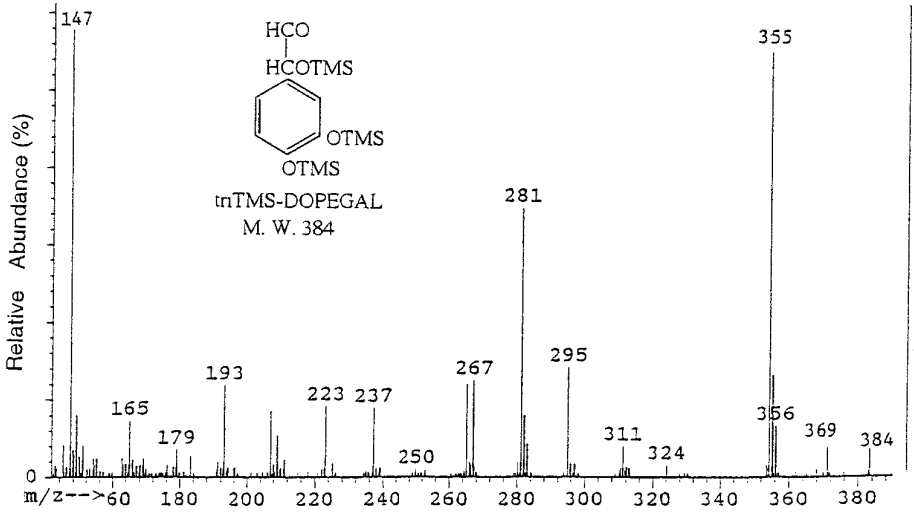

FIG. 2. Mass spectrum of tri-TMS derivative of unlabeled DOPEGAL.

ence of the other compounds bearing the catechol/benzylhydroxy structure is excluded. The chromatographic peak areas at retention time 11.3 min for ions at *m/z* 355 and 358 were integrated by computer and ratios of those areas calculated. After determination of appropriate area ratios and comparison to the calibration graph, the level of DOPEGAL in human brain tissue was found to be 2.2 ± 0.1 ng/mg wet tissue or 119 ng ± 5 ng/mg protein.

CONCLUSION

The synthesis of deuterated DOPEGAL is important for several reasons. We report here for the first time the quantitation of DOPEGAL in human locus ceruleus using GC–MS techniques. The human brain in a number of disorders, such as Parkinson's disease and Alzheimer's disease, is studied primarily using postmortem tissue. Deficits in catecholamines, including NA, are thought to play a role in these disorders (*12–14*). DOPEGAL is the precursor of the other major brain metabolites of NA, DOPEG, and 3-methoxy-4-hydroxyphenylglycol (MHPG) (*15*). Carlsson and Winblad have suggested that in postmortem brain the sum of a catecholamine plus its major metabolite is a better index of the level of catecholamine in brain at the time of death than the catecholamine alone (*16*). This is supported by the findings of Warsh *et al.* (*17*) that catecholamines are metabolized in brain after death. The GC–MS measurement of DOPEGAL will therefore contribute to a more accurate estimation of premortem NA levels in postmortem human brain. In addition, because the enzymes which metabolize DOPEGAL (aldehyde reductase, aldehyde dehydrogenase, and catechol-*O*-methyltransferase) either are in very low amounts (*18*) or are extraneuronal (*18*, *20*, *21*). DOPEGAL may be an index of

the intraneuronal metabolism of newly formed NA (*22*). The concentration of DOPEGAL reported here is comparable to that reported for MHPG, the major NA metabolite in rat LC (*11*).

The amount of LC DOPEGAL is 14 times that reported for NA and 57 times the reported value for MHPG in postmortem human LC (*10*). Several methodological differences may have led to this variation. The earlier report (*8*) used HPLC–EC instead of GC–MS to measure LC catechols. The mean delay between death and autopsy was 4.5 times as long as the PMI used here. In addition, there was a delay of up to 2 h prior to freezing at −70°C (*10*). In our study tissue was frozen immediately at autopsy and kept at −140°C. This difference is especially important in view of the report of further metabolism of catechols after death (*17*). Finally, because DOPEGAL is synthesized in neurons, it should be found primarily in neurons and not glial tissue. However, most of the tissue dissected is nonneuronal. Therefore, some variation in amounts of DOPEGAL expressed as ng/mg tissue could be due to differences in dissection technique. Regardless of these differences, our results indicate that DOPEGAL is a major NA metabolite in postmortem human brain.

DOPEGAL is the initial MAO-A metabolite of NA. Blashko (*23*) suggested that aldehyde metabolites of MAO action on amines may be toxic to cells in which they are formed. Markey *et al.* (*24*) have shown that the exogenous toxin 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine, which causes a form of Parkinson's disease, becomes toxic to neurons only after its oxidation to the pyridinium formed by MAO-B. We have shown that DOPEGAL is toxic to neurons both *in vivo* and *in vitro* (*25*). The concentration of DOPEGAL we report here in locus ceruleus tissue is only 2.3-fold lower than the concentration of 5.0 μg/ml which we found toxic to differentiated PC-12 cells (*25*). We have also reported a 2.2-fold increase in an enzyme in the biosynthetic pathway of DOPEGAL in C-1 epinephrine neurons (*26*) in Alzheimer's disease (AD). The synthesis of deuterated DOPEGAL thus allows us to determine if DOPEGAL has reached toxic levels in adrenergic neurons in AD.

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