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Fluorometric estimation of 4-hydroxy-3-methoxyphenylethyleneglycol sulphate in brain

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Summary

1. A simple, sensitive, and selective method was developed for the fluorimetric estimation of 4-hydroxy-3-methoxyphenylethyleneglycol sulphate (MOPEG-SO_4) in rat brain.

2. MOPEG-SO_4 was isolated on a DEAE Sephadex column. Fluorescence was measured at 465 nm (excitation at 320 nm) after heating the column eluate with acid and reheating after adding ethylene diamine.

3. The concentration in brain of MOPEG-SO_4 increased after blockade of its transport from brain by treatment with probenecid and decreased after inhibiting its synthesis by treatment with α-methyltyrosine or pargyline.

4. Our studies suggest that measurement in changes in the rate of formation of MOPEG-SO_4 may be a useful index of noradrenaline turnover.

Introduction

The compound, 4-hydroxy-3-methoxyphenylethyleneglycol sulphate (MOPEG-SO_4), is a major metabolite of noradrenaline catabolism in the brain of some species (Schanberg, Schildkraut, Breese & Kopin, 1968; Schanberg, Breese, Schildkraut, Gordon & Kopin, 1968). A simple, sensitive method for the estimation of MOPEG-SO_4 in brain would be useful since the rate of MOPEG-SO_4 formation may reflect the rate of noradrenaline formation. MOPEG and MOPEG-SO_4 have been assayed by gas chromatography (Wilk, Gitlow, Clarke & Paley, 1967; Sharman, 1969; Karoum, Ruthven & Sandler, 1971; Gordon & Oliver, 1971). MOPEG-SO_4 is enzymatically hydrolyzed overnight to form MOPEG. The MOPEG is partially purified by adsorption chromatography and finally a halogen-containing derivative is made which is quantitated using electron capture gas-liquid chromatography. MOPEG-SO_4 has been estimated in cerebrospinal fluid by a fluorometric method, but the originators doubted that their procedure could be used for brain samples (Korf, van Praag & Sebens, 1971). We have found that MOPEG-SO_4 can be readily isolated with an anion exchange column and that after heating in acid, MOPEG-SO_4 reacts with ethylene diamine to form a highly fluorescent compound. These findings allowed us to develop an assay for MOPEG-SO_4 that is about as sensitive as the gas chromatographic methods, but that is far more rapid and convenient.

Methods

Assay of MOPEG-SO_4

Rat brains were homogenized in 4 volumes of 0·2 M $ZnSO_4$ solution with a Polytron homogenizer (Brinkman Instruments, New York). We added 4 volumes

of 0·2 M $Ba(OH)_2$ solution to the homogenates and centrifuged them at 30,000 *g* for 10 minutes. The supernatant solution was passed through a 6 × 30 mm column of DEAE Sephadex A-25 (Pharmacia, Piscataway, N.J.). We discarded the effluent and then washed the column with 8 ml of 0·06 N HCl to remove weak anions. MOPEG-SO_4 was eluted from the column with 5·5 ml 0·15 N HCl.

We added 0·2 ml of 1·5% cysteine HCl in H_2O (made daily) and 0·3 ml of 60% perchloric acid to the 5·5 ml of the eluate. Two millilitre aliquots of the acidified eluate were used for sample, internal standard, and blank analysis. Two hundred pmol MOPEG-SO_4 (3-methoxy-4-sulphonyloxyphenylglycol, RO 4-6028, a gift from Hoffman La Roche, Nutley, N.J.) in 50 μl of water added to a sample served as an internal standard. Tubes containing the sample and standard were heated at 100° for 12 min in a water bath. Tubes for blank analysis were left at room temperature. Then we added 0·3 ml redistilled ethylene diamine to all the tubes, including the tubes containing the blanks, and heated them at 100° for 5 minutes. After cooling to room temperature, fluorescence at 465 nm was measured with an Aminco Bowman fluorometer (excitation 320 nm, Xe lamp), or with an Aminco SPF 125 fluorometer (excitation wavelength set at 305 nm, Hg lamp). The wavelengths given were uncorrected instrumental values.

Evaluation of the assay

Brains from 10 rats were pooled and homogenized and then divided into 10 portions. MOPEG-SO_4 (800 pmol) was added to 5 of the portions. MOPEG-SO_4 was then estimated as described. The possible contamination by weakly acidic compounds in the MOPEG-SO_4 eluate was examined by adding 7-^{3}H-4-hydroxy-3-methoxymandelic acid (New England Nuclear, Boston) (about 100,000 cpm) to each of 3 homogenates. Radioactivity in the MOPEG-SO_4 eluates from the Sephadex column was measured.

Compounds, structurally similar to MOPEG-SO_4, were heated with perchloric acid and ethylene diamine to examine the specificity of fluorophore formation.

The proportionality between fluorescence and MOPEG-SO_4 concentration was evaluated by adding various amounts of MOPEG-SO_4 to 5·5 ml of 0·15 N HCl and developing the fluorescence as described.

When the regional distribution of MOPEG-SO_4 was studied, rat brains (3 per sample) were dissected into 7 parts as described by Glowinski & Iversen (1966). Male, Sprague-Dawley rats, 180-210 g, obtained from Zivic Miller Labs. (Pittsburgh, Pa.) were killed by decapitation and their brain tissue was assayed immediately or frozen until analysed. In another study, we measured MOPEG-SO_4 concentration in brain 4 hours after inhibiting its formation by treatment with pargyline (75 mg/kg, i.p.), 2 h after inhibiting MOPEG-SO_4 transport from brain with probenecid (200 mg/kg, i.p.) or 4 h after blocking the formation of noradrenaline with α-methyltyrosine methyl ester (250 mg/kg, i.p.).

The MOPEG-SO_4 from rat brain was also identified in the 0·15 N HCl eluate by thin layer chromatography. A column eluate was adjusted to pH 7, and then freeze-dried. The residue was dissolved in methanol and applied to silica gel G plates. The plates (Analtech, Newark, Delaware, 250 μ thick) were pre-washed with methanol (ascending) to remove interfering fluorescent material. The extract and authentic MOPEG-SO_4 were chromatographed in butanol:pyridine:water

(14:3:3) or butanol:acetic acid:water (12:3:5). After drying the plates, 0·5 cm sections were scraped into tubes and 2 ml of water was added. The silica gel was removed by centrifugation and 0·1 ml 1% cysteine and 0·1 ml perchloric acid were added to each tube. Fluorescence was developed as above.

Results

The mean recovery of MOPEG-SO$_4$ added to a homogenate of brain was 82%. The S.E.M. of 5 determinations of the pooled homogenate was ±4·2% for 5 determinations. Less than 0·2% of a weakly acidic compound, 4-hydroxy-3-methoxymandelic acid, was recovered in the 0·15 N HCl eluate. The fluorescence spectra of MOPEG-SO$_4$ isolated from brain and authentic MOPEG-SO$_4$ coincided with each other (Fig. 1). Moreover, the apparent MOPEG-SO$_4$ extracted from brain and authentic MOPEG-SO$_4$ had similar R_f values after thin layer chromatography in butanol:acetic acid:water and butanol:pyridine:water, 0·45–0·50 and 0·55–0·60, respectively.

The fluorescence developed from MOPEG-SO$_4$ was proportional to concentration from 40 pmol to 40 nmol/5·5 ml of sample. About 40 pmol/5·5 ml of sample produced a fluorescence that was twice the blank value (Fig. 2). Of the structurally related compounds tested, only MOPEG produced an appreciable fluorescence with our procedure (Table 1). Indoxyl-SO$_4$ and 3,4-dihydroxyphenylalanine (DOPA) produced less than 2% of the fluorescence of an equimolar amount of MOPEG-SO$_4$. MOPEG and DOPA are not retained by the DEAE-Sephadex A-25 column and therefore would not interfere in the assay of MOPEG-SO$_4$. Indoxyl-

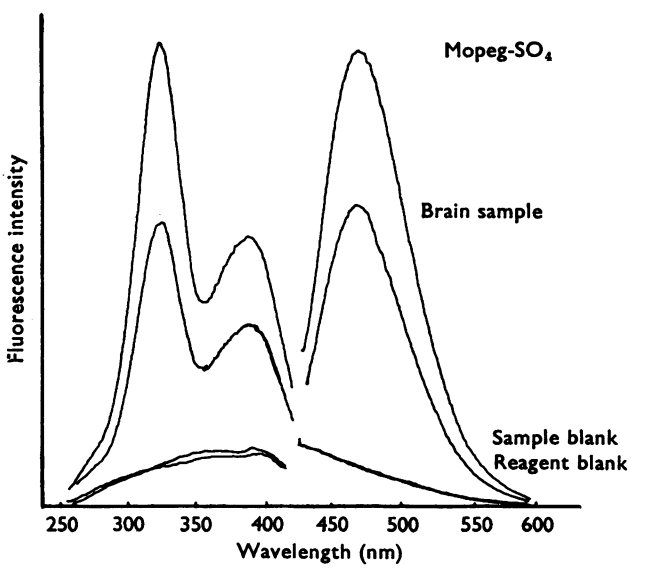

FIG. 1. Spectra of the fluorescence derived from 4-hydroxy-3-methoxyphenylethyleneglycol sulphate (MOPEG-SO$_4$) (400 pmol) and from MOPEG-SO$_4$ isolated from a rat brain. The excitation spectra were obtained with the emission at 465 nm; the emission spectra with the excitation at 320 nm.

SO_4, which is retained by the column, can be distinguished from MOPEG-SO_4 because it has a different fluorescence spectrum.

The regional distribution of MOPEG-SO_4 (Table 2) in brain paralleled that reported by Glowinski & Iversen (1966) for noradrenaline. Four hours after inhibiting

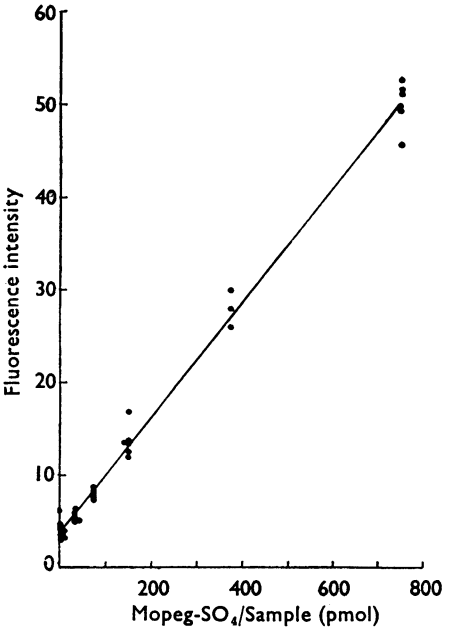

FIG. 2. Proportionality between concentration of 4-hydroxy-3-methoxyphenylethyleneglycol sulphate (MOPEG-SO_4) and fluorescence. After adding various amounts of MOPEG-SO_4 to 5·5 ml 0·1 N HCl, fluorescence was developed as described in **Methods.**

TABLE 1. *Relative fluorescence of 4-hydroxy-3-methoxyphenylethylene glycol sulphate (MOPEG-SO_4) and structurally related compounds*

	Fluorescence units
MOPEG-SO_4	100
MOPEG	104
Indoxyl SO_4	1·8
DOPA	0·8
Vanillin	0·5
3-methoxy, 4-hydroxyphenylethanol	<0·2
3-methoxy, 4-hydroxymandelic acid	<0·2
Homovanillic acid	<0·2
Normetanephrine	<0·2
3,4-dihydroxyphenylglycol	<0·2
3,4-dihydroxymandelic acid	<0·2
3,4-dihydroxyphenylacetic acid	<0·2
Dopamine	<0·2
Octopamine	<0·2
Tyramine	<0·2
Noradrenaline	<0·2
Tyrosine	<0·2
5-Hydroxyindoleacetic acid	0·3

Each of the above substances (0·1 μmol) was added to 2 ml of 0·4 N $HClO_4$ and the fluorescence determined (activation 305 nm; fluorescence 465 nm) after heating followed by condensation with ethylene diamine.

monoamine oxidase with pargyline, MOPEG-SO_4 concentrations had decreased. Two hours after blocking acid transport in brain with probenecid, MOPEG-SO_4 concentration had increased. Four hours after inhibiting tyrosine hydroxylase with α-methyl tyrosine treatment, MOPEG-SO_4 concentration had fallen.

Discussion

$ZnSO_4$ and $Ba(OH)_2$ were chosen to precipitate brain protein because only negligible quantities of ions from these substances remain in solution (unlike perchloric acid or trichloroacetic acid) to interfere with the subsequent isolation procedure. DOWEX anion exchange resins were unsatisfactory for the isolation procedure because of high blank fluorescence and the difficulty of elution of MOPEG-SO_4 from the resin. MOPEG-SO_4 can be readily separated from most non-anionic constituents of brain tissue with a DEAE Sephadex column. Weak anions can be removed from the column by washing with HCl. The amount of HCl which can be passed through the column without eluting MOPEG-SO_4 varies with different batches of DEAE Sephadex. Preliminary experiments showed that urine contains interfering substances which are not separated from MOPEG-SO_4 by the DEAE Sephadex column.

Korf *et al.* (1971) have enzymatically hydrolyzed MOPEG-SO_4 and then isolated the resulting MOPEG with a Sephadex G-10 column. Although MOPEG will form a fluorophore (Table 1), preliminary experiments with brain showed that our method of fluorescence development could not be used after their isolation procedure because of the interfering substances in the eluate. Our procedure might be used, however, if a more specific method for isolating free MOPEG were devised.

The structure of the fluorescent compound(s) formed by heating MOPEG-SO_4 with acid and then with ethylene diamine is unknown, but it seems likely that the sulphate moiety is lost by hydrolysis. Heating with perchloric acid, however, must cause additional chemical changes since free MOPEG will not produce a fluorophore with ethylene diamine unless heated first with acid. Both acid and ethylene diamine are required for the fluorophore formation, but cysteine is not. If cysteine

TABLE 2. *Concentration of 4-methoxy-3-methoxyphenylethyleneglycol sulphate (MOPEG-SO_4) in brain*

	MOPEG-SO_4 pmol/g Mean±S.E.M.	*n*
Whole rat brain	467± 7	4
After probenecid	770±58	4
After pargyline	122± 5	4
After α-methyltyrosine	316±14	4
Cerebellum	103±15	5
Cortex	248±56	5
Striatum	203±61	5
Hypothalamus	501±85	5
Medulla	567±33	5
Midbrain	201±27	5
Hippocampus	291±31	5

Rats (1/determination) were injected intraperitoneally with probenecid (200 mg/kg) 2 h before killing, pargyline (75 mg/kg) 4 h before killing or α-methyltyrosine (250 mg/kg) 4 h before killing. The regional distribution was determined with parts pooled from 3 brains. Values were corrected for recovery (82%).

is omitted from the tubes, fluorescence decreases to about a third. If the concentration of cysteine is increased above that used here, the fluorescence yield is also decreased. The optimal times of heating and the optimal concentrations of perchloric acid, ethylene diamine, and cysteine were determined in preliminary experiments. Heating MOPEG-SO_4 for 8 min in 1 N HCl (without cysteine) produces about the same fluorescence as when $HClO_4$ is used. However, the reaction in HCl was not satisfactory since the fluorescence yield varied with the batch of redistilled ethylene diamine.

Korf *et al.* (1971) measured MOPEG fluorimetrically (excitation 400 nm; fluorescence 500 nm) by heating with ethylene diamine, ammonia, and ammonium chloride after oxidation with potassium ferricyanide. For a blank reading, they added cysteine instead of the ferricyanide. The fluorophore formed with our procedure has different spectral characteristics and the development of fluorescence is not prevented by cysteine. Vanillin produced the same fluorophore as MOPEG with the procedure of Korf *et al.* (1971) suggesting that the ferricyanide probably oxidized the MOPEG to vanillin. In contrast, vanillin does not produce appreciable fluorescence with the present method (Table 1).

The presented evidence suggests that the fluorescent compound formed in the brain extracts is derived from MOPEG-SO_4: (1) the fluorescence spectra of the substances derived from authentic MOPEG-SO_4 and from the compound isolated from brain are identical; (2) structurally similar compounds known to occur in brain do not interfere with the assay; (3) thin layer chromatography of brain extracts revealed that the fluorophore is formed by a compound with the same R_f values as authentic MOPEG-SO_4 in two different solvent systems; (4) the adsorption characteristics of the material isolated from brain on DEAE Sephadex A-25 suggests that the substance is a strong anion rather than a weak anion; (5) pargyline, which inhibits an enzyme required for the formation of MOPEG-SO_4 from noradrenaline, lowered the concentration of MOPEG-SO_4 in brain; (6) probenecid, which inhibits the transport of MOPEG-SO_4 from brain (Meek & Neff, 1972) increased the concentration of MOPEG-SO_4 in brain; (7) blockade of noradrenaline formation with α-methyltyrosine lowered the concentration of MOPEG-SO_4 in brain; (8) the regional distribution of MOPEG-SO_4 parallels that of its precursor, noradrenaline; and (9) the values obtained for rat brain (467 pmol/g) are similar to those found by Schanberg *et al.* (1968) using a gas chromatographic method.

Our assay method for MOPEG-SO_4 is relatively simple and sensitive enough for routine measurements of MOPEG-SO_4 in rat brain. The changes of MOPEG-SO_4 concentrations in brain after treatment with drugs may be a useful index of noradrenaline turnover.

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