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DETERMINATION OF O-METHYLATION PRODUCTS OF NORADRENALINE

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SUMMARY

A method for the determination of normetanephrine, norparanephrine and 3,4-dimethoxyphenylethanolamine in aqueous solution is described. Periodate oxidation converts these O-methylated amines to aldehydes which are then extracted with benzene and separated by gas chromatography. Sensitivity is greatly increased if labeled amines are used.

The errors from the oxidation, extraction and chromatographic steps are reported for the unlabeled and labeled compounds. Special attention is given to the estimation of the O-methylated amines when they are derived from [^{14}C]noradrenaline in a mixture containing the competent enzyme.

An enzymatic preparation of [7-^{14}C]normetanephrine and [7-^{14}C]norparanephrine is also described.

INTRODUCTION

Although a mixture of normetanephrine (NM; 3-methoxy-4-hydroxy-β-phenylethanolamine) and norparanephrine (NP; 3-hydroxy-4-methoxy-β-phenylethanolamine) has never been completely resolved in its components, periodate oxidation converts NM and NP into the aldehydes vanillin (V) and isovanillin (IV) respectively, which have been separated by paper chromatography[1,2]. We show that, similarly, periodate oxidation of 3,4-dimethoxy-β-phenylethanolamine (DMPE) yields veratraldehyde (VA).

In this report, we describe an analytical method involving periodate oxidation, benzene extraction and gas chromatography, which determines the concentration of NM, NP and DMPE, the three possible O-methylation products of noradrenaline (NA). The accuracy of the method, which uses radioisotopes, is studied not only when the O-methylated amines are dissolved in water, but also when they are in the incubation mixture utilized for the assay of catechol-O-methyltransferase.

EXPERIMENTAL AND RESULTS

Vanillin, isovanillin and veratraldehyde separation by gas chromatography

An Aerograph Autoprep A-700 gas chromatograph equipped with a thermal

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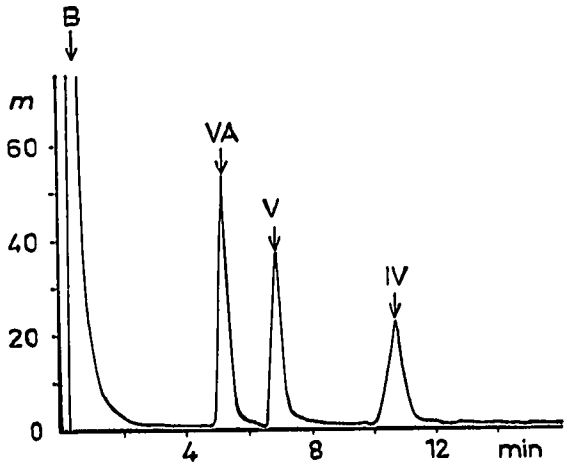

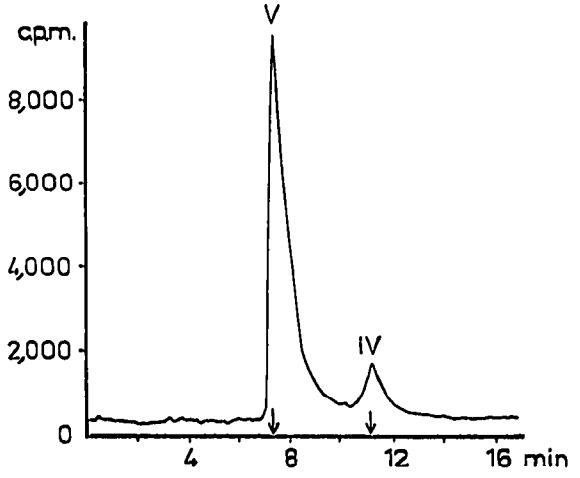

Fig. 1. Gas chromatographic separation of vanillin (V), isovanillin (IV) and veratraldehyde (VA). The injected mixture contains 120 μg of each aldehyde in a total volume of 50 μl of benzene (B) Mass (*m*) *vs.* elution time (min).

Fig. 2. Gas chromatographic separation of ^{14}C-vanillin (V) and ^{14}C-isovanillin (IV). 50 μl of benzene solution containing 15,000 d.p m. Radioactivity (c.p.m) *vs.* elution time (min). The arrows indicate the position of the mass peaks Recording conditions· detector operating voltage· 2100 V, time constant: 10 sec; sensitivity: 10,000 c.p.m. full scale, speed of recording chart· 25 mm/min.

conductivity detector was used. The column was a 180 × 0.35 cm steel tube; the stationary phase was a mixture of 1% HIEFF (Applied Science Laboratories) and 1% diethylene glycol succinate on Gas-Chrom Q (Applied Science Laboratories). Helium (60 ml/min) was used as the carrier gas; the injection port and the detector were at 220°, while the column temperature was maintained at 168°. Fig. 1 shows a typical separation following the injection of a benzene solution of a mixture of V (Fisher Scientific Co.), IV (K & K Laboratories) and VA (synthesized according to the method of PERKIN AND ROBINSON[3]).

When the aldehydes were radioactive, the helium eluent from the chromatographic column was mixed with methane (60 ml/min) before passing through the 200 ml chrome-plated brass gas-flow proportional detector of a Model 45 Gas Radiochromatograph Nuclear Chicago. The connecting tube was maintained at 225° and the detector at 250°. The gas-flow detector was coupled with a ratemeter and a recorder. The areas under the radioactivity peaks were measured by planimetry. Fig. 2 reproduces the radioactivity trace obtained after injection of a mixture of ^{14}C-V and ^{14}C-IV.

Between 15,000–20,000 d.p.m. in 50 μl benzene were required to obtain good tracings for planimetric measurements. As expected, Table I shows that, as the input d.p.m. decreases, the relative error increases.

Periodate oxidation of the unlabeled O-methylated amines and benzene extraction of the aldehydes

Standard procedure. Between 5–12 ml of an aqueous solution of NM, NP or DMPE was brought above pH 10.5 by the addition of concentrated NH_4OH. Then, a greater than ten-fold excess of $NaIO_4$ was added. After 10 min at room temperature, the solution was cooled to 0° in an ice bath and the pH lowered to 6.5 by the addition

TABLE I

REPRODUCIBILITY OF THE MEASURED ^{14}C-V/^{14}C-IV RATIO WHEN VARIOUS ALIQUOTS OF THE SAME SOLUTION ARE INJECTED SEVERAL TIMES

The solution contains 5 mg of each aldehyde and 825,000 d p m. per ml

Approximate injected volume (μl)	*Approximate radioactivity (d.p.m.)*	*Peak area*[a]		*Number of injections*	*V/IV ratio*
		V	*IV*		
45	37,000	75	6	6	13.1 ± 1.0
30	25,000	50	4	5	13 7 ± 1.0
20	16,000	34	2 5	4	14 3 ± 1.9
10	8,000	15	1.1	6	14 3 ± 4 6
5	4,000	7	<0 5	6	—

[a] Planimeter arbitrary units 1 cm^2 = 2.68 units.

of 10 *N* H_3PO_4. The cold solution was extracted by 10, 5, 5 and 5 ml of benzene, the organic phases combined and the total volume adjusted to 25 ml with benzene. The concentration of the aldehydes in the benzene solution was determined by gas chromatography by comparison with standard solutions.

Extraction yield. Between 25–30 mg of pure V, IV or VA were dissolved in 12 ml of 2.7 *N* NH_4OH containing 1% $NaIO_4$. Five determinations were performed for each aldehyde and the yield of the benzene extraction was 100 ± 5% for each of them.

Oxidation and extraction yield. Between 30–40 mg of NM·HCl (Calbiochem) or 5–6 mg of DMPE·HCl (synthesized in our laboratory) were dissolved in 2.5 ml of 3 *N* NH_4OH and oxidized by the addition of 5 ml of 5% $NaIO_4$. The yield with NM was 98 ± 5% (five determinations). With DMPE, two extractions with 5 ml of benzene each were sufficient (a third extraction gave no detectable VA, *i.e.* < 1%) and the volume was adjusted to 10 ml. The yield was 94 ± 5% (four determinations).

Oxidation and extraction of labeled O-methylated amines

(A) [7-^{3}H]Normetanephrine

7-^{3}H-NM (0.1 μCi; 23 mCi/mmole, NEC) was dissolved in 5 ml 3 *N* NH_4OH containing 0.4% $NaIO_4$. Benzene extraction was performed according to the standard procedure. The radioactivity of the benzene solution was determined in a Packard Tri-Carb spectrometer after addition of toluene and scintillators (PPO, dimethyl-POPOP); a [^{3}H]hexadecane standard was used to calculate the counting efficiency. The radiochemical yield of the oxidation–extraction procedure was only 79%. Therefore, the experiment was repeated with the addition of 30 to 40 mg of NM·HCl as a carrier, using 5% $NaIO_4$. The chemical yield, determined by gas chromatography, was 98%, while the radiochemical yield still remained around 80%. The missing label was found in the aqueous phase which lacked V.

The percentage loss of tritium was reproducible; seven experiments with 0.1 to 0.2 μCi of 7-^{3}H-NM gave a mean radiochemical yield of 83.6 ± 1.4%.

(B) Preparation of ^{14}C-labeled O-methylated amines

(i) [7-^{14}C]Normetanephrine. 0.2 μmoles of 7-^{14}C-NA (48 mCi/mmole; Schwartz Bioresearch) were incubated for 2 h at 37° with 0.7 ml 0.6 *M* phosphate buffer (pH 7.8),

5.0 ml of a catechol-O-methyltransferase preparation (ammonium sulfate fractionation of the supernatant from rat liver, followed by dialysis of the dissolved precipitate[4]), 100 μmoles $MgCl_2$, 10 μmoles (—)S-adenosyl-L-methionine (P.L. Biochemicals) and 5 μmoles cysteine·HCl (total volume = 7.0 ml). The reaction was terminated by the addition of 7 ml 5% trichloroacetic acid (TCA). The precipitate was centrifuged and the supernatant lyophilized. The lyophilization residue was then dissolved in 2 ml of 0.2 *M* ammonium acetate buffer (pH 6.1) and the solution was applied on a Rexyn 102 (H^+) column (35 × 2 cm) packed and equilibrated according to KIRSHNER AND MCGOODALL[5]. The column was eluted by 0.4 *M* ammonium acetate buffer (pH 5.2); four 4.0 ml fractions were collected every hour and the radioactivity of a 0.1 ml aliquot of each fraction was determined (Fig. 3).

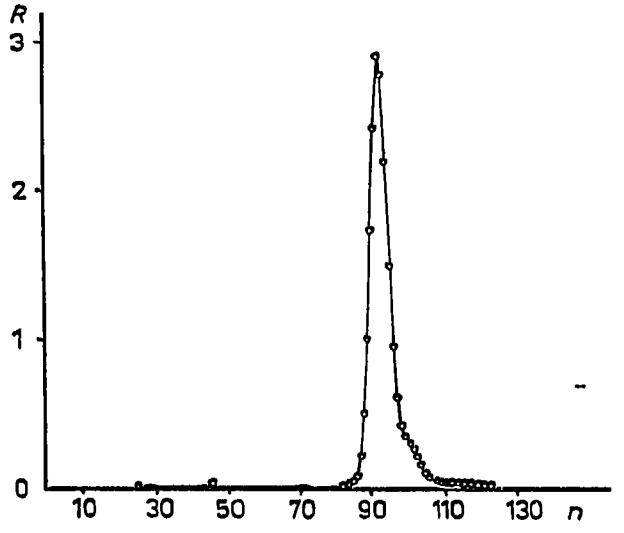

Fig. 3. Chromatography of the enzymatically synthesized ^{14}C-labeled normetanephrine and norparanephrine. Radioactivity (*R* in 10^6 d.p.m./0.1 ml) of fractions (*n*) eluted from the Rexyn 102 column. The main peak (maximum at 93) contains 7-^{14}C-NM, while the shoulder (beginning with fraction 100) also contains 7-^{14}C-NP.

Paper chromatography[6] of an aliquot of the pooled fractions 92–95 yielded only one radioactive spot which migrated at an R_F identical to pure NM. Periodate oxidation, benzene extraction and gas chromatography also yielded one radioactive peak, identified as V. From these results, we concluded that the pooled 92–95 fractions contained 7-^{14}C-NM with a radiochemical purity above 98%.

(ii) [7-^{14}C]*Norparanephrine.* Paper chromatography of the pooled fractions from the 100–102 Rexyn column yielded two radioactive spots. One spot (61% of the total radioactivity) migrated with an R_F identical to pure NM. This spot was eluted, an aliquot removed for immediate counting and another aliquot subjected to periodate oxidation and benzene extraction. The radiochemical yield of the oxidation-extraction was 92 ± 10%. Gas chromatography of the organic extract gave both radioactive V and IV, but the peaks were too small to be accurately measured.

The second spot (39% of the total radioactivity), eluted from the paper, was likewise treated. The radiochemical yield after oxidation and extraction was 12% and no radioactive V, IV or VA peaks were observed after gas chromatography. The second spot thus contained unidentified radiolabeled contaminants.

To determine the ratio of NM to NP, a sample of the pooled fractions 100–102 was oxidized by periodate, the solution was benzene extracted and the organic phase studied by gas chromatography. The radioactive peaks were measured and the ratio V/IV was found to be 0.23. Since the radioactivity of the mixture NM + NP amounted to 61% of the total, this ratio indicates that the ^{14}C of the pooled fractions 100–102 consisted approximately of 50% 7-^{14}C-NP, 11% 7-^{14}C-NM and 39% of unknown impurities. Further attempts to purify the 7-^{14}C-NM were unsuccessful because the amount involved was too small.

(C) Oxidation-extraction yield with 7-^{14}C-NM and 7-^{14}C-NP

Using 0.026 μCi of the 7-^{14}C-NM prepared in section *B (i)* in four different experiments, the oxidation-extraction yield was found to be 95 $\pm$ 1%. The impure preparation of NP described in section *B (ii)* was used to determine the yield of the oxidation-extraction procedure for ^{14}C-NP. In computing the yields, the radioactivity of the starting solution was corrected for the 39% of impurities observed by paper chromatography; the radioactivity of the organic phase was further corrected to account for the 12% of these impurities which were extracted in the organic phase. The combined yields for the labeled NP and NM were calculated to be 89 $\pm$ 2% (five experiments with 0.0294 μCi of the mixture, *i.e.* 0.0147 μCi of NP).

Determination of the labeled O-methylated amines in an incubation mixture containing catechol-O-methyltransferase

(A) The standard mixture used in the experiments with catechol-O-methyltransferase contained, in a total volume of 2.5 ml, 2 ml of 0.06 *M* sodium phosphate buffer (pH 7.8), 5 mg of proteins, 2 μmoles $MgCl_2$, 2 μmoles (—)S-adenosyl-L-methionine, 0.1 μmole cysteine·HCl. After addition of the labeled O-methylated amine, 2.5 ml 5% TCA were added. The precipitate was collected by centrifugation and washed twice with 1.3 ml 2.5% TCA. To the three combined supernatants, the following reagents were added: 5 μg NM·HCl, 10 μg NA·bitartrate hydrate, 1.25 ml concentrated NH_4OH and 1 ml 1% $NaIO_4$ (the quantity of periodate must be adjusted to the quantity of substances to be oxidized). After 30 min, the mixture was cooled to 0° in an ice bath, the pH adjusted to 6.5 and the solution extracted according to the standard procedure. Table II shows that, for 7-^{14}C-NM (0.025 μCi), the yield was about 94%.

(B) When determining catachol-O-methyltransferase activity, the incubation mixture also contains unreacted ^{14}C-NA. Five experiments with 0.11 μCi of 7-^{14}C-NA as the only labeled compound in the incubation mixture showed that 0.6 $\pm$ 0.06% of the periodate degradation products contaminated the organic phase.

The yields of oxidation-extraction for ^{14}C-NM and ^{14}C-NP were then studied in the presence of ^{14}C-NA; the values, corrected for the contamination by NA degradation products, appear in Table II.

(C) In most cases, when the labeled O-methylated amines were produced by the enzyme from ^{14}C-NA, the benzene solution of the radioactive aldehydes was too dilute for direct chromatographic analysis. Therefore, a concentration step was added.

The solution containing a mixture of ^{14}C-NM and ^{14}C-NP was oxidized with periodate and extracted with benzene. The radioactivity of an aliquot of the organic phase was determined and 100 μg of V, IV and VA were added per 20,000 d.p.m. to

TABLE II

RADIOCHEMICAL YIELD AFTER OXIDATION OF 7-^{11}C-NM AND 7-^{14}C-NP PRESENT IN CATECHOL-O-METHYLTRANSFERASE STANDARD INCUBATION MIXTURE AND BENZENE EXTRACTION

Substance	*Quantity (μCi)*	*Number of experiments*	*7-^{14}C-NA (μCi)*	*Yield (%)*
7-^{14}C-NM	0.025	5	0	93.8 ± 1.7
	0.0068 to 0.0135	13	0.11	93.2 ± 2.1
7-^{14}C-NP	0.0147[a]	5	0 54	85 ± 3.5

[a] 0.0294 μCi of the impure preparation.

the organic phase which was then dried on sodium sulfate and concentrated by vacuum distillation to have 400,000 d.p.m. per ml. 50 μl were injected in the gas chromatograph; mass and radioactivity tracings were simultaneously recorded. The ratio of the areas under the V and IV radioactivity peaks gave the NM/NP ratio in the primary solution.

The aldehydes evaporated during the concentration process, but it was shown by the mass peaks that the ratio V/IV did not change.

DISCUSSION

NM, NP and DMPE concentrations in aqueous solution are determined by periodate oxidation to V, IV and VA, followed by benzene extraction and gas chromatographic separation of the aldehydes. Trace amounts of these O-methylated amines can be determined if they are labeled. Addition of carrier aldehydes to the benzene solution and simultaneous records of mass and radioactivity in the effluent from the chromatographic column allow the identification of the radioactive peaks and the estimation of their isotope content by planimetric measurement of their areas.

Above pH 10.5, the yield of the periodate oxidation of milligram amounts of NM into V or of DMPE into VA is at least 95%. At pH 6.5 at 0°, the aldehydes (V, IV, VA) are quantitatively extracted from aqueous solutions into benzene. At a lower pH or a higher temperature, the yield of the oxidation step decreases due to the increasing oxidizing power of the periodate.

When using 7-^{3}H-NM, the radiochemical yield of the oxidation into 7-^{3}H-V is lower (79%) than the chemical yield (98%). As the ^{3}H-C bond is stable both in NM and V, the exchange of the ^{3}H must take place during the oxidation process itself, probably at the level of the intermediary complex. Although the loss of ^{3}H was reproducible (16.4 ± 1.4%), we nevertheless decided to use the ^{14}C-labeled amines instead.

7-^{14}C-NM and 7-^{14}C-NP were prepared enzymatically from 7-^{14}C-NA. The final 7-^{14}C-NM was radiochemically pure (more than 98%), while we obtained a preparation of 7-^{14}C-NP whose radiochemical purity was only 50%. It was shown with 7-^{14}C-NM that the radiochemical yield of oxidation into V was the same as the chemical yield. The presence of impurities in the 7-^{14}C-NP preparation necessitated corrections in the computation of the yield of oxidation into IV; nevertheless, the computed radiochemical yield closely agrees with the experimental data obtained for 7-^{14}C-NM.

The yield for ^{14}C-DMPE could not be verified because this compound was unavailable. However, as the chemical yield for the oxidation of the unlabeled amine is about 95%, it is very probable that the radiochemical yield for the labeled product will be of the same order of magnitude.

The three aldehydes (V, IV, VA) can be separated by gas–liquid chromatography. The separation is completed in less than 15 min and the amount of radioactivity associated with each aldehyde can be computed from the areas of their respective peaks. This procedure can thus be used to estimate the *para* and *meta*-O-methylated, and the *meta,para*-O-dimethylated products eventually obtained by the action of catechol-O-methyltransferase or other enzyme preparations on ^{14}C-NA.

When the benzene solution is concentrated before the chromatographic analysis, a loss of the aldehydes occurs, but the ratio V/IV remains unchanged; thus, the ^{14}C-NM/^{14}C-NP can still be determined. As the radioactive assay before concentration yields the NM + NP value, it is therefore possible to determine ^{14}C-NM and ^{14}C-NP separately in the incubation mixture.

Since periodate oxidation of the O-methylated derivatives of adrenaline (metanephrine, paranephrine and N-methyl DMPE) also yields the same aldehydes (V, IV, VA respectively), this method may also be applicable if adrenaline serves as substrate for the enzyme; however, the method will not permit to differentiate between the products obtained from the two catecholamines if both are present unless they are labeled with different isotopes and the proportional counter equipped with a two channel analyzer. 3-Methoxy-4-hydroxymandelic (VMA) and 3-hydroxy-4-methoxymandelic (iso-VMA) acids also yield V and IV on periodate oxidation[7] so that the method can also be used to determine these two compounds.

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