Br. J. Pharmac. (1975), 53, 143-148

THE ESTIMATION OF 3,4-DIHYDROXYPHENYLACETIC ACID, HOMOVANILLIC ACID AND HOMO-ISOVANILLIC ACID IN NERVOUS TISSUE BY GAS–LIQUID CHROMATOGRAPHY AND ELECTRON CAPTURE DETECTION

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1 A gas chromatographic method using electron capture detection is described for the estimation of three acidic metabolites of dopamine, 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-hydroxy-4-methoxyphenylacetic acid (homo-isovanillic acid, iso-HVA). The method is based on the formation of the trifluoroacetyl-hexafluoroisopropyl derivatives of the three acids.

2 The method has been applied to the estimation of DOPAC, HVA and iso-HVA in tissues from the central and peripheral nervous systems.

Introduction

Methods for the estimation of 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA) based on the formation of fluorescent derivatives have been used to study the effects of drugs on the metabolism of 3,4-dihydroxyphenylethylamine (dopamine) in the brain. However, these fluorimetric methods are not sensitive enough to enable changes in the concentrations of these two acidic metabolites of dopamine to be measured in small, discrete regions of the central nervous system of laboratory rodents without pooling tissues from several animals. This paper describes a more sensitive method for the estimation of the acid metabolites of dopamine based on the formation of the hexafluoro-isopropyl esters of their trifluoroacetyl derivatives (a procedure first described by Dziedzic, Bertani, Clarke & Gitlow in 1972) and their estimation by gas-liquid chromatography (GLC) combined with electron capture detection.

Methods

Materials

Ethyl acetate: Reeve Angel Scientific Ltd, CT grade. If required dry, this was dried over calcium hydride and distilled twice.

Trifluoroacetic anhydride (TFA): BDH Chemicals Ltd. This reagent was distilled twice using a 10 cm fractionating column filled with Fenske helices and was then stored at -17°C in sealed glass ampoules each containing sufficient trifluoroacetic anhydride for one set of samples (usually 1.5 ml). Any reagent remaining in an ampoule was discarded.

1,1,1,3,3,3-Hexafluoroisopropanol (HFIP): Pierce Chemical Co. This reagent was distilled once using a 20 cm long Fenske helix fractionating column and stored under dry N_2 at room temperature.

Diethyl ether: Reeve Angel Scientific Ltd, CT grade. Boron trifluoride 99.5%: BDH Chemicals Ltd.

Boron trifluoride etherate. Approximately 1 ml diethyl ether was placed in a test tube and boron trifluoride was passed into it through a polytetrafluoroethylene tube until the diethyl ether was saturated.

4-Hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA): Calbiochem and Sigma. 3-Hydroxy-4-methoxyphenylacetic acid (homo-isovanillic acid, iso-HVA), prepared by the method of Grundon & Perry (1954), m.p. 129.5-130°C uncorrected. 3,4-Dihydroxyphenylacetic acid (DOPAC): Sigma, recrystallized from benzene.

Pentafluorophenol: Koch-Light Ltd. Distilled once under vacuum.

Benzoyl chloride: May & Baker Ltd.

Other chemicals were of analytical reagent quality.

Gas-liquid chromatography

This was carried out with Pye model 104 gas-liquid chromatographs fitted with [^{63}Ni] electron capture detectors. The carrier gas was argon containing 5% or 10% methane. Several liquid phases coated on Diatomite CQ were tested and these

together with column and detector oven temperatures and gas flow rates are given with the results. These columns were cured at 250° C with the carrier gas flowing through them for at least 16 h before use.

Reaction vials

The bases of small screw capped glass vials (0.5 dram series SNB, Camlab Ltd) were blown to form round bottomed reaction vials. The plastic caps were fitted with a disk of clear silicone rubber (Esco Rubber) 1.5 mm thick and a disk of polytetrafluoroethylene 0.25 mm thick which ensured an inert gas-tight seal.

Evaporations

These were always carried out in a thermostatically controlled metal heating block.

Preparation of crystalline derivatives of HVA, DOPAC and iso-HVA

Approximately 0.5 g of the parent acid was added to a mixture of 5 ml trifluoroacetic anhydride and 2.5 ml hexafluoroisopropanol. This was usually refluxed overnight but a reasonable yield of the HVA derivative can be obtained after 5 hours. The trifluoroacetic anhydride and hexafluoroisopropanol were removed at room temperature under vacuum and the remaining liquid was distilled under vacuum. The HVA derivative distilled at 120-130° C at approximately 8 mmHg pressure (water pump). The product was a clear, colourless, viscous liquid which crystallized when stored at −17° C but was liquid near room temperature (m.p. 27-30° C uncorrected). The DOPAC derivative distilled at 84-88° C at 0.1 mmHg pressure yielding a clear colourless viscous liquid which crystallized on cooling (m.p. 44-45° C uncorrected). The iso-HVA derivative distilled at 95-97° C at 0.3 mmHg pressure and also gave a clear, colourless, viscous liquid which crystallized when stored at −17° C and melted at 31-33° C (uncorrected).

Preparation of pentafluorophenyl benzoate

Pentafluorophenyl benzoate (PFPB) was found to be a suitable substance to serve as a standard in determining the concentrations of DOPAC, HVA and iso-HVA in tissue extracts by the peak height ratio method (Vandenheuvel, Hinderks, Nixon & Layng, 1965; Wisniewski & Umbreit, 1965).

Pentafluorophenol 1.0 g was dissolved in 5 ml 10% w/v NaOH solution cooled in an ice bath; 1.0 ml benzoyl chloride was added slowly and the mixture then shaken vigorously for 5-10 min with intermittent cooling. A white semi-crystalline solid formed and was extracted into dichloromethane. The extract was dried over anhydrous sodium sulphate and evaporated to dryness under vacuum. The white crystalline solid which remained was dissolved in warm hexane, the solution was filtered and then cooled to 0° C. The crystals (m.p. 73-75° C uncorrected) which formed were filtered off in a cold filter funnel and then dried on filter paper until no smell of benzoyl chloride could be detected.

Extraction of tissues

Two methods of precipitating tissue proteins have been used. The first, using zinc sulphate and barium hydroxide, was found to be suitable for the estimation of HVA alone and the second, using perchloric acid followed by potassium chloride to remove the perchlorate ion from solution, enabled the estimation of iso-HVA and DOPAC in addition to HVA.

Method 1. The estimation of HVA in nervous tissues

Up to 250 mg tissue was frozen in liquid nitrogen, crushed in a stainless steel die if necessary and then homogenized in 1.0 ml 0.2 M zinc sulphate solution. The homogenate was transferred to a centrifuge tube together with 1 ml water used to wash the homogenizer. To this, 1.0 ml of 0.2 M barium hydroxide solution was added and mixed thoroughly. (The zinc sulphate and barium hydroxide solution must be titrated against each other before use with phenol phthalein as indicator, to ensure equal normalities.) The mixture was centrifuged and the clear supernatant was transferred to a glass test tube containing 0.5 g potassium chloride and 0.1 ml 1 N HCl. Ethyl acetate (4.0 ml) was added and the HVA extracted by mixing for 1 min on a vortex mixer. The phases were separated by centrifugation and 3.5 ml of the ethyl acetate was taken and evaporated under dry N_2 at 50° C to a volume of approximately 0.2 ml. This was transferred to a reaction vial and the tube washed with approximately 0.1 ml ethyl acetate which was also added to the vial. The ethyl acetate extract was then evaporated to dryness under dry N_2 at 50° C. The vial was removed from the heating block as soon as all of the ethyl acetate had disappeared; 0.2 ml of trifluoroacetic anhydride was added to the reaction vial followed by 0.1 ml hexafluoroisopropanol containing 10% v/v freshly prepared boron trifluoride etherate. The reaction vial was tightly closed, the contents mixed and it was then heated at 100° C in a metal

heating block for 15 minutes. The estimation was unsatisfactory if the reaction vial leaked during this heating. The reaction vial was then allowed to cool to room temperature before opening and the contents were evaporated under a stream of dry N_2 at room temperature until a small amount of an oily brown residue remained. This was dissolved in 0.5-1.0 ml of dry ethyl acetate containing a known concentration of pentafluorophenyl benzoate (20-50 ng/ml).

In the early experiments to determine the optimal conditions for the reaction, it was observed that a low recovery of the derivative of HVA was obtained if the boron trifluoride etherate was omitted from the reaction. However, it was later found with tissue extracts that a satisfactory recovery could be obtained when the boron trifluoride was omitted if the heating was continued for 60 min and the final evaporation was stopped as soon as all of the liquid had disappeared. Most of the results have been obtained without using boron trifluoride etherate because fewer peaks are seen on the chromatograph tracings when this reagent is omitted. It seems that the presence of the boron trifluoride etherate resulted in a material with a high boiling point which retained the volatile HVA derivative in the reaction vial thus preventing the loss seen with pure solutions which was presumably due to evaporation of the HVA derivative.

Method 2. The simultaneous estimation of HVA and DOPAC

The procedure now used routinely is as follows:

1. Dissect tissue (up to 250 mg) and freeze at once in liquid N_2. Crush in stainless steel die if necessary.
2. Homogenize in 1.0 ml 0.1 N HCl, add 1.0 ml H_2O and mix.
3. Add 25 µl 72% w/v perchloric acid; mix.
4. Add 0.5 g KCl, mix and then centrifuge in homogenizer tube at 5,000 x *g* for 3 min at room temperature.
5. Carefully transfer (avoid transfer of fatty material) the supernatant to a cellulose nitrate centrifuge tube containing 0.5 g KCl; mix.
6. Freeze in liquid N_2 until solid. Allow to thaw until liquid is just visible around a frozen pellet. Centrifuge at 15,000 x *g* for 15 min at 10° C. This step was found to be necessary for consistent recoveries.
7. Transfer supernatant to a glass test tube containing a few crystals of KCl. Add 4.0 ml ethyl acetate and mix on a vortex mixer for two periods of 30 s each. The tubes are placed in ice between the two mixes while the contents of the other tubes in the series are mixed. Centrifuge at 4,000 x *g* for 1 min to separate the phases.
8. Transfer 3.5 ml of the organic upper layer to a glass test tube and evaporate at 50° C under a stream of dry N_2 until approximately 0.2 ml remains. This is transferred to a reaction vial and the tube washed with 0.1 ml ethyl acetate which is also transferred to the reaction vial. Evaporate the ethyl acetate to dryness at 50° C under a stream of dry N_2.
9. Add 0.2 ml trifluoroacetic anhydride and 0.1 ml hexafluoroisopropanol to the reaction vial and close tightly. Heat at 100° C for 1 hour.
10. Allow reaction vial to cool to room temperature before opening and then evaporate contents *just* to dryness at room temperature under a stream of dry N_2. Dissolve the residue in a measured volume of dry ethyl acetate containing a known amount of pentafluorophenyl benzoate for injection into the gas chromatograph. Routinely the residue is dissolved in 1.0 ml of dry ethyl acetate containing 50 ng/ml pentafluorophenyl-benzoate; 0.2-2 µl of this solution is injected into the gas chromatograph.

Extraction of cerebrospinal fluid and vitreous humour

Pig cisternal cerebrospinal fluid (0.5 ml) was diluted to 2 ml with 0.1 N HCl in a cellulose nitrate centrifuge tube; 25 µl perchloric acid was added and mixed, followed by 1.0 g potassium chloride. After thorough mixing to ensure saturation with the potassium chloride the solution was frozen solid in liquid N_2, partially thawed, centrifuged at 15,000 x *g* for 15 min and the supernatant treated as above. Aqueous and vitreous humour from goat and pig eyes was extracted in a similar manner, the amounts of reagents used in the extraction of the phenolic acids being increased approximately in proportion to the volume of body fluid used.

Results

The retention times of the TFA-HFIP derivatives of DOPAC, HVA and iso-HVA relative to that of PFPB are shown in Table 1 and show that clear separations of the derivatives can be obtained. The ratios of the peak heights of the derivatives of DOPAC and HVA to that of PFPB showed a linear relation with the amounts of the derivatives of DOPAC and HVA in a sample (prepared from crystalline derivatives) relative to the amount of PFPB present in the same sample. Thus the peak height ratio method could be used to quantify the

amounts of HVA and DOPAC present in extracts of tissues.

The recovery of HVA (50 ng) added to homogenates of mouse brain and estimated after protein precipitation with zinc sulphate and barium hydroxide gave a mean recovery (±s.e. mean) of 79 ± 6% (n = 28) when estimated with a 3.8% S.E.30 column and 65 ± 5% (n = 28) when the same samples were estimated with a 2% QF1 column. It was found that of the three types of column tested, that with a stationary phase of 2% S.E.52 was the most suitable for the simultaneous estimation of HVA and DOPAC. The recovery of DOPAC and HVA (25-50 ng) added to homogenates of mouse brain tissue and estimated with a 2% S.E.52 column was 62 ± 3% (mean ± s.e. mean; n = 51) and 65 ± 4% (n = 59) respectively.

The reproducibility of the method was tested by estimating the concentration of HVA and DOPAC in replicate portions of homogenates of mouse striatal tissue. Most duplicate estimates have shown reasonable reproducibility and in 11 experiments in which the estimation of HVA was made in triplicate or quadruplicate the mean coefficient of variation was 8.9% (range 0.9-14.8%) and in two experiments in which DOPAC was estimated in triplicate the coefficients of variation were 11.8% and 12.9%.

The specificity of the method has been tested by estimating the concentration of HVA in mouse brain tissue with two different chromatography columns and by comparing the values obtained by the GLC method for the concentrations of HVA and DOPAC in different tissues with the values which have been obtained by fluorimetry.

Table 2 gives estimates of the concentrations of DOPAC and HVA obtained with the present GLC method in different nervous tissues and body fluids.

When the HVA in the striatal tissue of mice was

Table 1 The retention times of the trifluoroacetyl-hexafluoroisopropyl (TFA-HFIP) derivatives of acid metabolites of dopamine relative to that of pentafluorophenyl benzoate

Column length and liquid phase	*Column temperature (°C)*	*Relative retention time*		
		TFA-HFIP DOPAC	*TFA-HFIP HVA*	*TFA-HFIP iso-HVA*
2.75 m; 2% S.E. 52	115	0.35	0.52	0.58
	95	0.37	0.55	0.63
1.5 m; 2% QF 1	105	1.52	1.36	
1.5 m; 3.8% S.E. 30	105	0.36	0.52	

The carrier gas was argon containing 5% or 10% methane with a flow rate of 40 ml/min at pressures of 15-20 kg/cm^2. The detector temperature was 220°C.

Table 2 The concentration of 3,4-dihydroxyphenylacetic acid (DOPAC) and 4-hydroxy-3-methoxyphenylacetic acid (HVA) in some tissues and body fluids

Species	*Tissue or body fluid*	*Concentration (μg/g or *μg/ml)*	
		DOPAC	*HVA*
Rat	Caudate nucleus	2.25 ± 0.37 (9)	0.58 ± 0.06 (9)
	Olfactory tubercle	1.14 ± 0.15 (5)	0.52 ± 0.04 (5)
Mouse	Striatum	0.17 ± 0.01 (13)	0.26 ± 0.02 (13)
Pig	Cisternal cerebrospinal fluid	0.014 ± 0.002 (4)*	0.060 ± 0.001 (4)*
Goat	Vitreous humour	0.038; 0.085*	0.021; 0.012*
	Retina	0.008; 0.005†	0.004; 0.004†
Rabbit	Superior cervical ganglion	not estimated	0.42 ± 0.04 (4)
Sheep	Caudate nucleus	0.94 ± 0.18 (3)	6.7 ± 1.8 (3)

Values are given as mean with s.e. mean and are uncorrected for recovery. The number of observations is in parentheses. † Content in μg/retina, 2-4 retinae were pooled for each estimation.

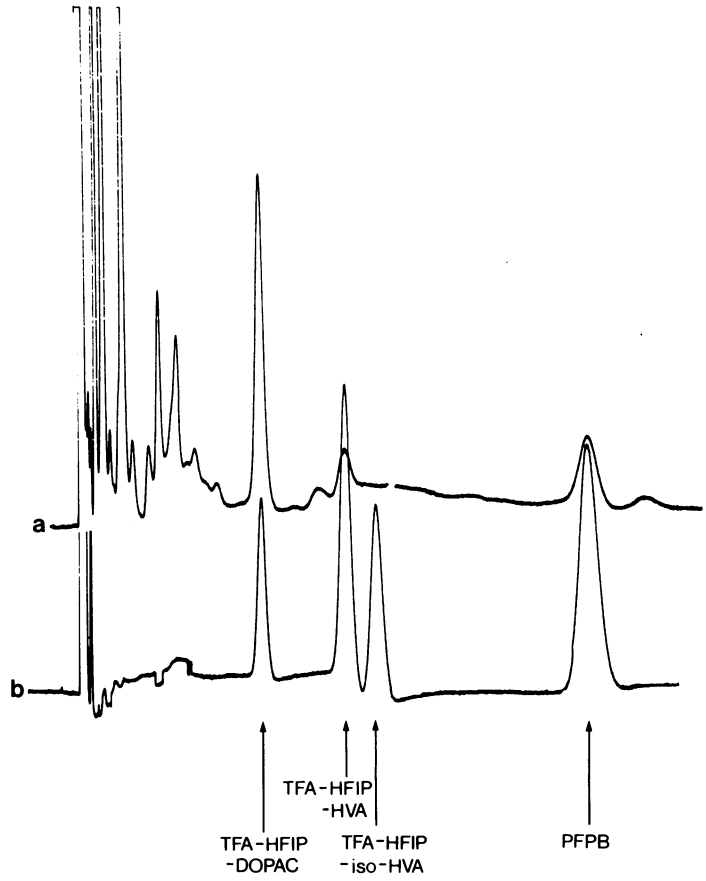

Figure 1 Gas chromatographic record obtained with (a) an extract of rat striatum compared with (b) standards. The arrows indicate the retention times of the trifluoroacetyl-hexafluoroisopropyl derivatives of 3,4-dihydroxyphenylacetic acid (TFA-HFIP-DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (TFA-HFIP-HVA), 3-hydroxy-4-methoxyphenylacetic acid (TFA-HFIP-iso-HVA) and pentafluorophenyl benzoate (PFPB). The column was 2.75 m long packed with 2% S.E.52 coated on Diatomite CQ. Oven temperature 115° C. Detector temperature 220° C. Carrier gas argon: 5% methane; flow rate 40 ml/min at 20 kg/cm^2.

estimated on both the 3.8% S.E.30 and 2% QF1 columns the values obtained were not significantly different ($t = 0.25$; $n = 8$; paired t test) although the relative retention times differ greatly on the two columns. The estimates of the concentrations of HVA and DOPAC in the mouse striatum given in Table 2 were obtained with 2% S.E.52 columns. The values obtained compare well with those reported by Sharman (1966, 1967) for HVA and Roffler-Tarlov, Sharman & Tegerdine (1971) for DOPAC, and were similar to the values obtained in this laboratory by fluorimetric methods during the period when the GLC estimates were made. A chromatograph record of an extract of rat striatum is given in Figure 1. The present estimates for the concentrations of the two acids in the rat caudate nucleus confirm the observations of Guldberg & Broch (1971) that the major metabolite of dopamine present in rat striatal tissue is DOPAC.

Although both HVA and DOPAC could be estimated in the vitreous humour of the goat, the application of the GLC method to aqueous humour met with a major difficulty. There was a substance present in extracts of aqueous humour which had a retention time relative to PFPB very close to that of the derivative of DOPAC. However, no DOPAC (<10 ng/ml) could be detected in the same sample of aqueous humour when a fluorimetric method (Murphy, Robinson & Sharman, 1969) was used for its estimation. No HVA could be detected in aqueous humour.

The GLC method will also permit the con-

current estimation of iso-HVA (Dziedzic, Dziedzic & Gitlow, 1973; Dziedzic & Gitlow, 1974). However, in all of the samples which we have examined, except for sheep caudate nucleus, no evidence for the presence of iso-HVA was obtained. With extracts of sheep caudate nucleus small peaks were observed with a relative retention time identical with that of TFA-HFIP-iso-HVA. These gave an estimate of the concentration of iso-HVA in sheep caudate nucleus of 0.8 ± 0.3 μg/g ($n = 3$), equivalent to 12% of the concentration of HVA in this tissue.

Discussion

The present method for the estimation of HVA and DOPAC is applicable to a wide range of tissues. Dziedzic *et al.* (1973) and Dziedzic & Gitlow (1974) have demonstrated that the GLC method is suitable for the estimation of HVA in human urine and cerebrospinal fluid and the present experiments show that it is possible to extend this to the estimation of DOPAC.

The limit of sensitivity is about 1 ng of HVA or DOPAC in a tissue sample which is similar to that reported for HVA in cerebrospinal fluid by Dziedzic & Gitlow (1974). However, like most methods for the estimation of very small amounts of substances its specificity, although good, is not absolute. This is illustrated by the presence of an unknown substance in extracts of aqueous humour which might be mistaken for DOPAC. It is thus advisable always to examine extracts of tissues using a second chromatography column on which the relative retention times of the derivatives are different and preferably show a different sequence of elution or to examine a pooled sample of the tissue under investigation by fluorimetry.

The method allows studies of the formation of the acid metabolites of dopamine in smaller samples of brain tissue than has been possible by fluorimetric techniques and may enable more precise observations to be made on the role of dopamine in the central nervous system.

The only tissue in which iso-HVA was detected was the sheep caudate nucleus. This confirms the observation of Mathieu, Charvet, Chazot & Trouillas (1972) that iso-HVA is present in the sheep caudate nucleus. The present estimate of the concentration (12% of the HVA present) is much lower than that found by their chemical method of assay (53-58%); however, the three sheep examined were of different breeds and the concentrations varied widely.

The authors would like to thank the Medical Research Council for financial support.

References

DZIEDZIC, S.W., BERTANI, L.M., CLARKE, D.D. & GITLOW, S.E. (1972). A new derivative for the gas-liquid chromatographic determination of homovanillic acid. *Analyt. Biochem.*, **47**, 592-600.

DZIEDZIC, S.W., DZIEDZIC, L.B. & GITLOW, S.E. (1973). Separation and determination of urinary homovanillic acid and iso-homovanillic acid by gas-liquid chromatography and electron capture detection. *J. Lab. clin. Med.*, **82**, 829-835.

DZIEDZIC, S.W. & GITLOW, S.E. (1974). Cerebrospinal fluid homovanillic acid and iso-homovanillic acid: a gas-liquid chromatographic method. *J. Neurochem.*, **22**, 333-335.

GRUNDON, M.F. & PERRY, H.J.H. (1954). Bisbenzylisoquinolines. Part 1. The synthesis of 4-(2-aminoethyl)-5'-carboxymethyl-2:2'-dimethoxydiphenyl ether and phenoxyisoquinolines. *J. Chem. Soc., Lond.*, 3531-3537.

GULDBERG, H.C. & BROCH, O.J., JR. (1971). On the mode of action of reserpine on dopamine metabolism in the rat striatum. *Eur. J. Pharmac.*, **13**, 155-167.

MATHIEU, P., CHARVET, J.C., CHAZOT, G. & TROUILLAS, P. (1972). Les métabolites 4-O-méthyles des catécholamines: L'acide 3-hydroxy,4-méthoxyphénylacetique (acide homoiso-vanillique, iso-HVA) dans les milieux biologiques: urine, liquide cephalorachidien, tissu cérébral. *Clinica chim. Acta*, **41**, 5-17.

MURPHY, G.F., ROBINSON, D. & SHARMAN, D.F. (1969). The effect of tropolone on the formation of 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the brain of the mouse. *Br. J. Pharmac.*, **36**, 107-115.

ROFFLER-TARLOV, S., SHARMAN, D.F. & TEGERDINE, P.R. (1971). 3,4-Dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the mouse striatum: a reflection of intra- and extra-neuronal metabolism of dopamine? *Br. J. Pharmac.*, **42**, 343-351.

SHARMAN, D.F. (1966). Changes in the metabolism of 3,4-dihydroxyphenylethylamine (dopamine) in the striatum of the mouse induced by drugs. *Br. J. Pharmac. Chemother.*, **28**, 153-163.

SHARMAN, D.F. (1967). A discussion of the modes of action of drugs which increase the concentration of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) in the striatum of the mouse. *Br. J. Pharmac. Chemother.*, **30**, 620-626.

VANDENHEUVEL, F.A., HINDERKS, G.J., NIXON, J.C. & LAYNG, W.G. (1965). Precise ultramicro determination of steroid hormones by combined TLC and GLC analysis. *J. Am. Oil Chem. Soc.*, **42**, 283-290.

WISNIEWSKI, J.V. & UMBREIT, G.R. (1965). Gas chromatographic determination of sapogenins. A reliable method for analysing steroid-like compounds of plant origin. *Facts and Methods for Scientific Research*, **6**, 9-10.

(Received July 8, 1974)