ON THE USE OF AN ACETYLATED DERIVATIVE OF NORMETANEPHRINE FOR GAS CHROMATOGRAPHIC ANALYSIS

JEAN-MARIE FRÈRE* AND WALTER G. VERLY
Department of Biochemistry, Montreal University, P.O.Box 6128, Montreal (Canada)
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Using the gas chromatography technique, HORNING *et al.*[1] have separated the acetylated derivatives of adrenaline, noradrenaline, metanephrine and normetanephrine. This qualitative performance seemed to contain the promise of a quantitative method of estimating small amounts of normetanephrine (α-[3-methoxy-4-hydroxyphenyl]-β-aminoethanol) in the presence of noradrenaline (α-3,4-dihydroxyphenyl-β-aminoethanol), separation and assay being done in a single operation.

We have prepared the tri-acetylated derivative of normetanephrine in a pure, crystalline form. This standard was used to study the yield of formation of triacetylnormetanephrine during the acetylation process and also to check the accuracy and sensitivity of normetanephrine assay by gas chromatography after acetylation. We further analyzed the influence of the presence of noradrenaline on the acetylation of normetanephrine.

EXPERIMENTAL AND RESULTS

(1) *Gas chromatography*

Gas chromatography was used as the main analytical tool to follow all the steps of the present study.

We use an F and M Research chromatograph Model 810, with a flame ionization detector.

The columns are prepared in the following way: 49.6 g of Gas Chrom Z (80-100 mesh) are suspended in the warm solution resulting from the mixing of 0.1 g of cyclohexanedimethyl succinate in chloroform and 0.3 g of dimethylpolysiloxane in ethyl acetate. The solvents are evaporated and the columns are filled. After an overnight baking at 275° in a current of helium, the columns are ready for use.

The compound is dissolved in ethyl acetate and the volume of the injected solution never exceeds 50 μl. The elution gas is helium (50 ml/min). After a 5 min equilibration at 165°, the temperature program climbs from 165° to 225° at a rate of 4° per min.

The column must be replaced after a continuous use of 4 weeks because of a slow bleeding of the liquid phase. During this aging process, the elution temperature of a given compound decreases (4 to 5° in 4 weeks); moreover the elution temperature is slightly different from one column to another of the same age. These difficulties are circumvented by the use of a standard of the pure compound.

* Aspirant du Fonds National Belge de la Recherche Scientifique.

(2) Preparation and isolation of the acetylated derivative of normetanephrine eluted at 218°

We tried HORNING's acetylation technique[1] and found that it was too mild and gave a poor yield in acetylated derivative. Moreover, the process, being carried out in the presence of air, gave a high amount of oxidation impurities.

To obtain the compound in a high yield, we had to modify the procedure in the following way:

100 mg of normetanephrine hydrochloride are dissolved in 20 ml of a warm mixture of acetonitrile, acetic anhydride and pyridine (10:3:3; v/v). The tube containing this solution is sealed after evacuation of the air, and heated overnight at 110°. After evaporation, the oily residue is dissolved in ethyl acetate; the boiling solution is treated by active charcoal and filtered. The residue of the evaporated filtrate is recrystallized in a mixture of petroleum ether and ethyl acetate.

The white crystals, dried under vacuum in the presence of P_2O_5, melt at 128–129° ((corr.)). Paper chromatograms did not detect any contaminant. The compound was found to be at least 98% pure by gas chromatography; the little peak at 212° (Fig. 1) remained unexplained: it persisted unchanged after several recrystallisations.

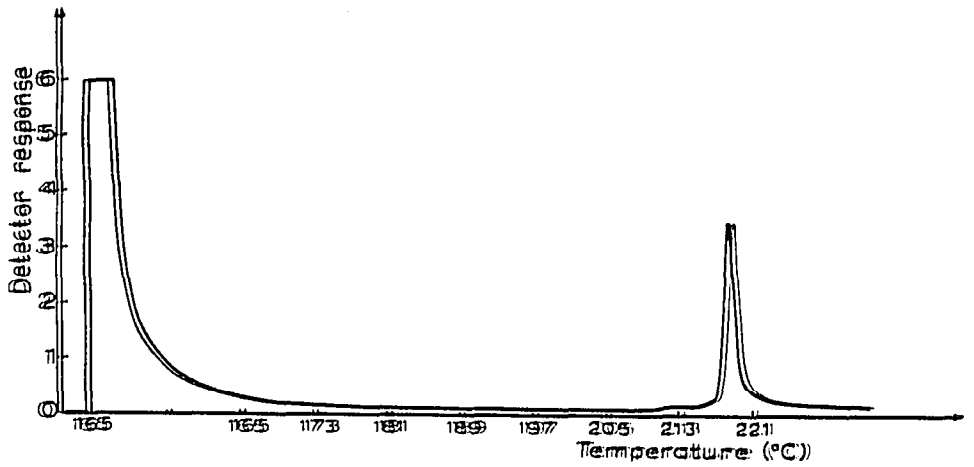


Fig. 1. Gas chromatogram of 100 μg of the crystalline derivative of normetanephrine dissolved in ethyl acetate.

(3) Identification of the acetylated derivative of normetanephrine

The problem is to know how many acetyl groups have been fixed onto the normetanephrine.

The interpretation of the elementary analysis remains doubtful (Table I).

Assay, according to the method described by NIEDERL AND NIEDERL[2], gave 3.62 ± 0.10 acetyl groups per molecule, but a control with the base normetanephrine

TABLE II

ELEMENTARY ANALYSIS OF THE ACETYLATED DERIVATIVE OF NORMETANEPHRINE

	Diacetylated derivative	*Triacetylated derivative*	*Found*
% C	58.44	58.27	58.24
% H	6.37	6.15	6.25

yielded 0.52 ± 0.04. It thus seems that, under the conditions used, the normetanephrine is oxidized and produces volatile acids which are titrated along with the acetic acid. We are probably entitled to correct the first figure given above to reach a value of 3.10 ± 0.14 acetyl groups per molecule.

To check this result, tritium labelled acetic anhydride was used to acetylate normetanephrine under the conditions described in paragraph 2, and also some β-naphthylamine. The acetylated normetanephrine and the β-naphthylacetamide were recrystallized to constant specific radioactivity. The activity of the naphthylacetamide, $5.85 \cdot 10^7$ d.p.m. per millimole, was taken as the activity of the acetyl milliequivalent. The specific activity of the acetylated derivative of normetanephrine was $6.18 \cdot 10^4$ d.p.m. per mg, from which result one can calculate that the compound contains 3.26 acetyl groups per molecule. The radioactivity determinations introduce a standard error of about 2 % on this final result.

The compound shows two infrared absorption bands in the region of the CO groups, one around 1700 cm^{-1} is characteristic of an amide group, the other around 1750 cm^{-1} depends on ester functions but, even on a Perkin-Elmer 112 spectrometer of high resolution (single beam, double pass), it cannot be dissociated into an alcohol and a phenol ester (Fig. 2). The absence of an OH band between 3500 and 3600 cm^{-1} is however in agreement with the hypothesis that the compound is a triacetyl derivative of normetanephrine.

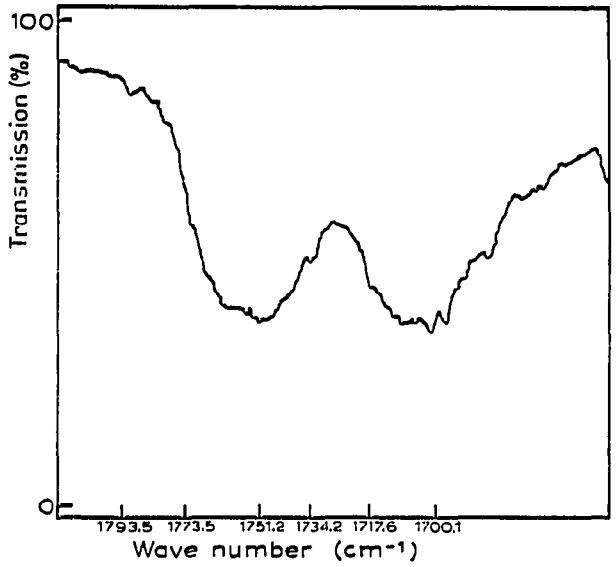


Fig. 2. I. R. spectrum of a 10 % solution in chloroform of the acetylated derivative of normetanephrine (Perkin-Elmer 112, single beam, double pass).

The esterification of the phenol OH is also confirmed by the action of pH on the U.V. spectrum: with normetanephrine, a rise of pH shifts the absorption maximum from 280 to 286 mμ with a considerable decrease of intensity; no such modifications are observed with the prepared acetylated derivative (Fig. 3).

Magnetic nuclear resonance spectra indicate the presence of four CH_3 groups distributed in three bands: methoxy, acetamide and one with a double area corresponding to the two acetyl esters (Fig. 4).

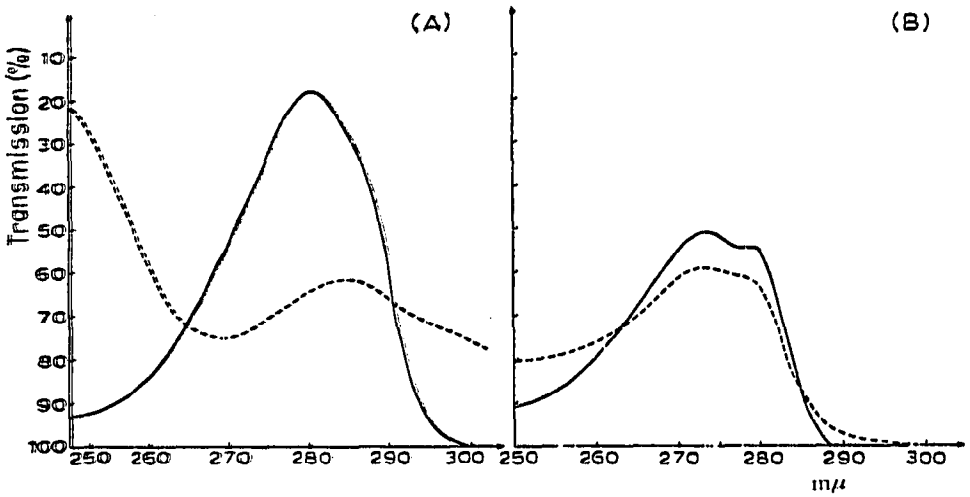


Fig. 3. Action of pH on the U.V. spectra of normetanephrine (A) and the acetylated derivative of normethanephrine (B). Continuous line = normetanephrine hydrochloride or triacetyl-normetanephrine dissolved in ethanol; dotted line = after addition of aqueous sodium hydroxide.

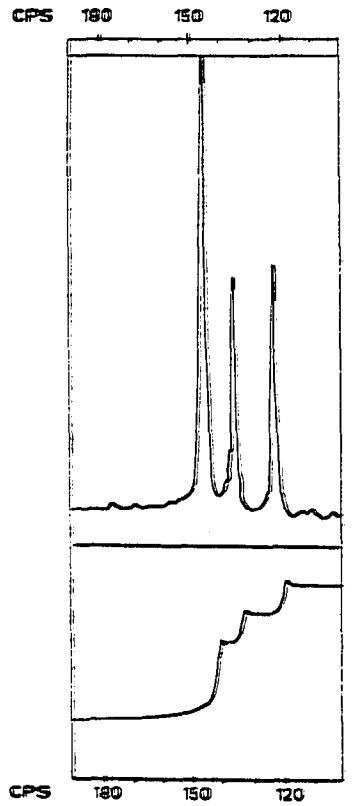
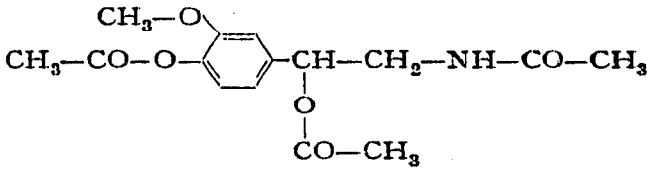


Fig. 4. Magnetic nuclear resonance spectrum of the acetylated derivative of normetanephrine dissolved in chloroform (above) and integration of the peak areas (below).

We believe that the phenol OH has been acetylated and that the isolated compound is the triacetyl derivative of normetanephrine of the following formula:



(4) *Kinetics and yield of the acetylation reaction*

40 mg of normetanephrine hydrochloride are dissolved in 8 ml of warm acetylating mixture (acetonitrile, acetic anhydride, pyridine). 1 ml aliquots are sealed in tubes *in vacuo*, and heated at 110° for periods varying from 0 to 60 min. After suitable dilution with ethyl acetate of the tube content, 10 μl are injected into the chromatographic column.

At time 0, there is only one peak eluted at 224°. Later, the area of this first peak decreases, while another peak appears at 218°, position taken by the standard of triacetylated normetanephrine (Fig. 5). It is likely that the 224° peak corresponds to the diacetyl derivative of normetanephrine with amine and secondary alcohol acetylated, the heating being necessary only for the acetylation of the phenol function.

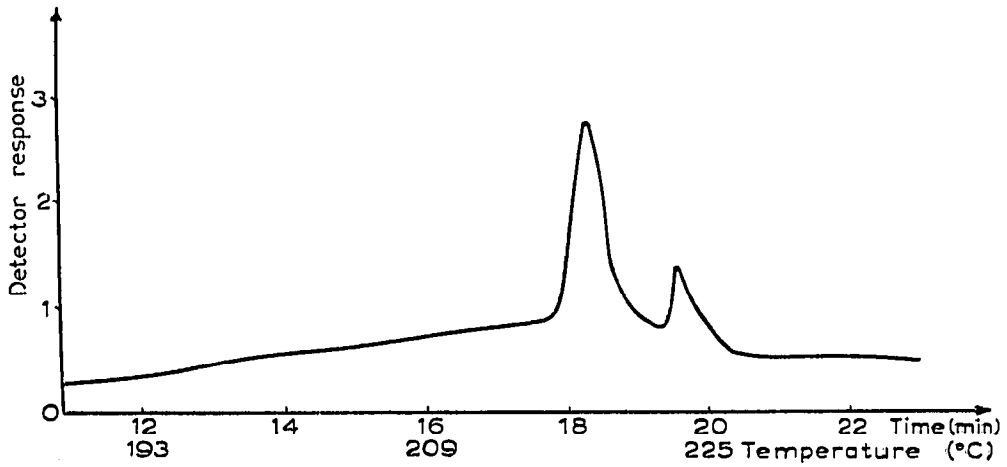


Fig. 5. Acetylation of normetanephrine: gas chromatogram of the acetylating mixture after a 30 min heating at 110°.

Fig. 6 gives the kinetics of growth of the 218° peak and disappearance of the 224° peak. The acetylation is completed within 4 h and the utilization of the standard shows that the formation of the triacetyl derivative is quantitative: 13 experiments gave a mean of 99 % with a standard deviation of 2 %, using 1 to 10 mg of normetanephrine hydrochloride as starting material.

(5) *Accuracy and sensitivity of the method*

(a) In a first set of experiments, the purified triacetylated derivative of normetanephrine was dissolved in ethyl acetate and small aliquots of the solution were injected in the chromatograph.

When the determinations are performed consecutively on the same day, the standard deviation for a single injection was 1 % with 5 or 10 μg, 3 % with 2 μg and 6 % with 1 μg of the compound (Table II).

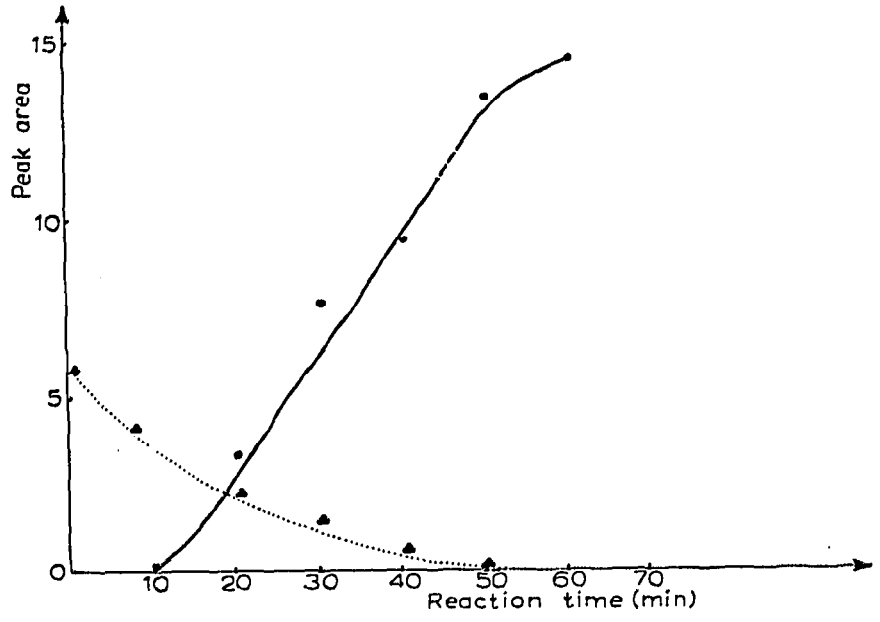


Fig. 6. Acetylation of normetanephrine. Continuous line = growth of the 218° peak (triacetylated derivative of normetanephrine); dotted line = disappearance of the 224° peak (likely the diacetyl derivative).

TABLE II

ACCURACY OF TRIACETYL-NORMETANEPHRINE DETERMINATION BY GAS CHROMATOGRAPHY

In each experiment, the same quantity was injected six times in the column on the same day. The standard deviation for one injection (S) could be calculated from the surfaces of the 6 peaks determined with a planimeter.

Experiment No.	*Attenuation*	*Amount* (μg)	*Mean surface*	S	$\frac{S}{surface}$	$\frac{Surf. \times att.}{amount}$
1	16	10	26.93	0.29	1.1%	43.0
2	16	10	25.07	0.28	1.1	40.2
3	16	5	14.69	0.25	1.7	47.0
4	16	5	12.45	0.22	1.8	39.9
5	8	5	24.79	0.31	1.25	39.7
6	4	2	17.32	0.52	2.9	34.7
7	2	1	15.64	0.86	5.5	31.3

But the results were very different from one day to another (compare experiments 1 and 2, or 3 and 4; Table II). It is thus of no use to plot a calibration curve of the product (peak area × attenuation) *versus* (amount of substance). The technique rather requires the unknown and a similar quantity of the standard to be injected immediately one after the other.

(b) The error in the assay of normetanephrine, instead of its acetylated derivative, was studied in a second set of experiments. Preliminary acetylation was thus required.

When the amount to be acetylated was of the order of 1 mg, so that a small and easily known aliquot of the reaction mixture diluted with ethyl acetate could be injected in the chromatograph, the chemical process did not add any supplementary error.

When the amount to be acetylated was of the order of 10 μg, the following procedure was used: the reaction medium (0.5 ml of the mixture of acetonitrile, acetic anhydride and pyridine + the amine), after 8 h heating at 115° in a sealed tube, was evaporated *in vacuo* to an oily residue which was dissolved in 5 ml of ethyl acetate; this solution was brought down to dryness. The residue was taken in ethyl acetate and filtered, the filtrate reduced to about 20 μl, most of which was injected in the column. There were two difficulties: the assessment of the exact portion of the whole

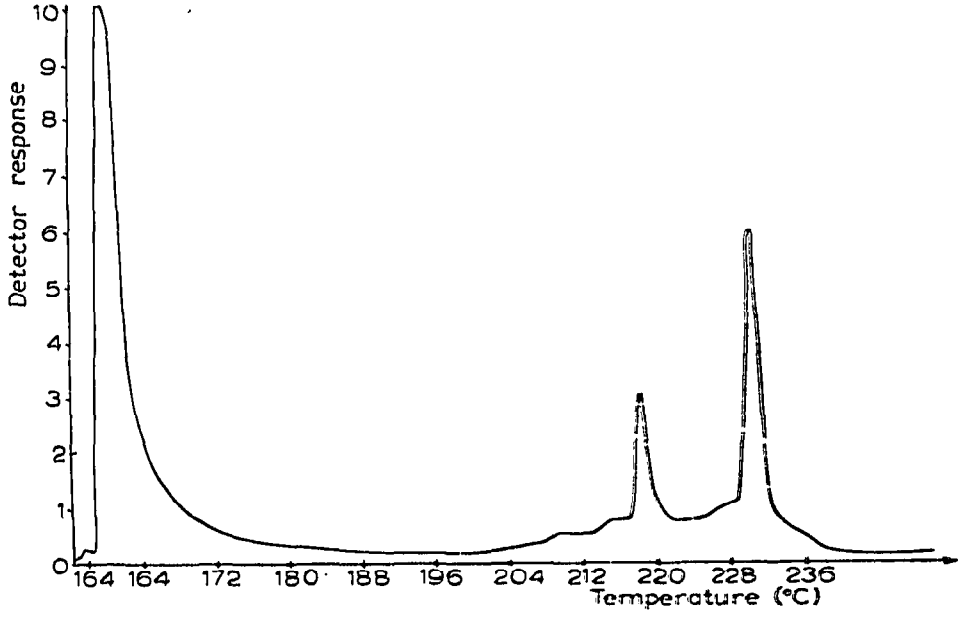


Fig. 7. Gas chromatogram of the acetylation products of a mixture of 1.8 mg of normetanephrine and 25 mg of noradrenaline. The 5 μl aliquot injected in the chromatograph represents 1/1000 of the whole.

used for the chromatographic assay; the concentration of products which were neither ethyl acetate nor the acetylated derivative of normetanephrine. The addition of an internal standard of methyl palmitate, prior to the evaporation of the reaction medium, solved the first problem. For the second one, although all the reagents had been purified and redistilled, the concentrated impurities made the base line of the chromatogram unreliable.

(6) *The presence of noradrenaline*

When a mixture of noradrenaline and normetanephrine are acetylated for 5 h under ordinary conditions, one gets two peaks in the gas chromatogram: 218° and 230°; this confirms the observation of HORNING *et al.*[1]. The 230° compound is given by noradrenaline with a very poor yield; moreover, the presence of noradrenaline decreases the formation of the triacetylated normetanephrine in an erratic manner (yields varying between 29 and 42 %).

DISCUSSION AND CONCLUSIONS

The noradrenaline and normetanephrine must be separated before a quantitative assay of normetanephrine, after complete acetylation, by gas chromatography can be considered.

Although the gas chromatography offers a means of measuring 1 μg of triacetyl-normetanephrine with a good accuracy, one is limited by the fact that the sample must be in a few μl. When one starts from normetanephrine that must be acetylated prior to chromatographic assay, the method is easily applicable only to mg amounts of the substance, which is of very limited physiological interest.

While this work was under way, CAPELLA AND HORNING[3] reported the preparation of trimethylsilyl derivatives of biological amines. Their study is only qualitative and has been done with mg amounts of the amines; it does not solve the difficulties which have been described in this paper.

ACKNOWLEDGEMENT

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SUMMARY

(1) The triacetylated derivative of normetanephrine has been prepared and identified. The white crystals melt at 128–129° (corr.).

(2) The compound is suitable for gas chromatographic analysis and can be prepared from normetanephrine in a quantitative manner.

(3) The presence of noradrenaline decreases the yield of production of triacetyl-normetanephrine and makes it erratic, so that a quantitative assay of normetanephrine by this method requires a previous separation of noradrenaline and normetanephrine.

(4) Although one can detect 1 μg of triacetyl-normetanephrine with a good accuracy, the whole procedure—acetylation and gas chromatography—cannot be applied to measure very small amounts of normetanephrine.

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3 P. CAPELLA AND E. C. HORNING, *Anal. Chem.*, 38 (1966) 316.