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## DERIVATIZATION OF CATECHOLAMINES IN AQUEOUS SOLUTION FOR QUANTITATIVE ANALYSIS IN BIOLOGICAL FLUIDS

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### SUMMARY

The reaction of methyl chloroformate with catecholamines at the nanogram level in aqueous solution has been estimated. Optimal pH conditions of the aqueous medium were established which provide a quantitative yield of the formate derivative of primary and secondary catecholamines. First the catechol function was blocked by the reaction with methyl chloroformate under mild alkaline conditions (pH 7.2) and subsequently a pH shift was introduced ( $\Delta$  pH = 2) to improve the reaction of the amine function. The formate derivatives were extracted effectively (> 99%) into ethyl acetate and subsequently converted to their *O*-*tert*-butyldimethylsilyl, *N*-formate derivatives. These mixed derivatives appeared to be very suitable for quantitative determination of catecholamines and related compounds in biological fluids by gas chromatography—mass spectrometry. The coefficient of variation estimated in urine samples was 8% ( $n = 6$ ). The minimal detectable concentration in biological samples was 50 pg ml<sup>-1</sup> with a signal-to-noise ratio of 5.

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### INTRODUCTION

The physiologically important catecholamines occur in low concentrations in body fluids. Apart from the difficulty in assessing small amounts, the analysis of catecholamines is hindered by their sensitivity to oxidative degradation and their amphoteric character. These properties necessitate a careful

sample-handling procedure under very strict conditions. Conventional sample clean-up methods use ion-exchange column chromatography [1] or adsorption to aluminium oxide [2] or boric gel [3]. An essential disadvantage of these methods is the risk of irreversible adsorption [4], which becomes especially serious if very small amounts have to be isolated. Recently, ion-pair extraction of catecholamines [5] and related compounds [6] has been described. This appears to be a very promising method, but the recovery depends on many factors which differ for each individual compound [6]. Brooks and Horning [7] demonstrated the potential utility of acetylation in aqueous media in the isolation of amines from dilute aqueous solutions. This method is based on the fact that amines and phenols easily react with anhydrides [8, 9] and alkyl chloroformates [10–12]. The stable acetyl or carbamate derivatives can be extracted effectively into relatively nonpolar solvents and easily concentrated. Thereafter, compounds containing only phenolic hydroxy groups or amino groups can be analyzed by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) directly from the extract. However, compounds containing additional aliphatic hydroxy groups will in general demand a subsequent derivatization because of their poor chromatographic properties.

In this paper the reaction of methyl chloroformate with the different functional groups of catecholamines in buffered aqueous media is described. The lowest pH values were determined at which the catecholic function is completely blocked by the reaction with methyl chloroformate. Subsequently the pH of the medium can be increased to values necessary for quantitative reaction of the amine function.

In addition, a number of silylation methods were evaluated for their utility in the formation of a singular mixed trialkylsilyl-formate derivative. *tert.*-Butyldimethylsilylation (tBDMS) turned out to be the most versatile silylation method. The tBDMS reagent reacts with the free aliphatic hydroxy group together with a complete substitution of the O-methyl formate group by an O-tBDMS group. The resulting O-tBDMS, N-carbamate derivatives are extremely stable and very useful for the GC–MS determination of catecholamines at physiological concentrations.

## MATERIALS AND INSTRUMENTATION

### *Chemicals*

Epinephrine (E), norepinephrine hydrochloride (NE), metanephrine hydrochloride (MN), normetanephrine hydrochloride (NMN), dopamine hydrochloride (DA) and isoprenaline bisulfate (IP) were obtained from Sigma (St. Louis, MO, U.S.A.). 1,2-Dihydroxybenzene was obtained from Merck (Darmstadt, G.F.R.) and 3,4-dihydroxybenzylamine from Aldrich Europe (Beerse, Belgium). Methyl chloroformate for synthesis (95%) was available from Merck. The silylating reagents hexamethyldisilazane (HDMS), N,O-bis-(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane (TMCS) were supplied by Pierce (Rockford, IL, U.S.A.) and trimethylsilylimidazole (TSIM) by Merck. *tert.*-Butyldimethylchlorosilane (1 mmol)–imidazole (2.5 mmol) in dimethylformamide (1 ml) was obtained from Applied Science Labs. (State

College, PA, U.S.A.). The normal alkanes hexadecane, tetracosane, octacosane and triacontane were obtained from Polyscience (Niles, IL, U.S.A.)

All solvents were of analytical grade.

### *Buffers*

Phosphate buffers used were 1 M  $K_2HPO_4$  in distilled water, the pH being adjusted with concentrated phosphoric acid (Merck). The sodium carbonate buffer was a saturated solution of sodium carbonate in distilled water, the pH adjusted with 12 N sodium hydroxide to pH 11.

### *Instrumentation*

*Gas chromatography.* GC analyses were carried out on a Hewlett-Packard 5810 A instrument equipped with a flame ionization detector. The chromatographic column was a fused-silica capillary column 25 m  $\times$  0.22 mm I.D., coated with CpSil-5 (Chrompack, Middelburg, The Netherlands). The chromatographic conditions were: injector temperature 250°C, split ratio 30:1, carrier gas (helium) flow-rate 28 cm sec<sup>-1</sup>. The oven temperature was varied in various experiments.

*Gas chromatography-mass spectrometry.* GC-MS analyses were performed on a Jeol JMS-D100 instrument with JMA 0231 data system. The chromatographic column was an OV-1701 fused-silica capillary column, 25 m  $\times$  0.22 mm I.D. (Chrompack). The oven temperature was 270°C, injection port temperature 300°C, and GC-MS interface temperature 270°C. The capillary column was directly introduced into the ion source of the mass spectrometer. Samples were introduced by a modified solid injector [13]. The MS conditions were: source temperature 250°C, electron energy 70 eV, and emission current 0.3 mA.

## METHODS

### *pH optimization experiments*

*Reaction with phenolic hydroxy groups.* Solutions of  $10^{-3}$  M 1,2-dihydroxybenzene in 0.02 N hydrochloric acid (0.5 ml), and 1.0 ml of phosphate buffer of a pH ranging from 6.25 to 8.0 were mixed with 25  $\mu$ l of methyl chloroformate for 10 sec and allowed to react for 5 min. Then the derivatives were extracted into 2 ml of ethyl acetate (containing hexadecane, 100  $\mu$ g ml<sup>-1</sup>, as internal standard) by shaking for 5 min; 1  $\mu$ l of the extracts was analyzed by GC at an oven temperature of 160°C.

Determination of the rate of the reaction of methyl chloroformate with phenolic hydroxy groups was performed in analogous experiments, but in these experiments the pH of the phosphate buffer was kept at 7.5 and extractions were carried out at 1, 5, 10 and 30 min after addition of methyl chloroformate.

*Reaction with primary amines.* The yield of the reaction of methyl chloroformate with primary catecholamines was determined for dopamine. Volumes ranging from 0.03 to 0.5 ml of  $2 \times 10^{-3}$  M dopamine in 0.02 N hydrochloric acid were made up to 0.5 ml with 0.02 N hydrochloric acid. These solutions were mixed with 1.0 ml of phosphate buffer (pH 7.5) and 25  $\mu$ l of methyl chloroformate. After 5 min the derivatives were extracted into 2 ml of ethyl

acetate, containing tetracosane ( $100 \mu\text{g ml}^{-1}$ ) as internal standard. The extracts were analyzed by GC at an oven temperature of  $240^\circ\text{C}$ .

*pH shift.* For secondary amines a pH shift was introduced. The influence of the pH of the aqueous medium on the rate of reaction of secondary catecholamines with methyl chloroformate was determined for epinephrine ( $1 \mu\text{g}$ ) and isoprenaline ( $1 \mu\text{g}$ ) with norepinephrine ( $1 \mu\text{g}$ ) as internal standard. To  $0.5 \text{ ml}$  of this mixture in  $0.02 \text{ N HCl}$  were added  $1.0 \text{ ml}$  of phosphate buffer (pH 7.5) and  $25 \mu\text{l}$  of methyl chloroformate, and after mixing allowed to react for  $5 \text{ min}$ . Then a volume of saturated sodium carbonate ( $0\text{--}1.0 \text{ ml}$ ) of pH 11 and a second volume of  $25 \mu\text{l}$  of methyl chloroformate were added and vigorously mixed. After  $10 \text{ min}$  the derivatives were extracted into  $2 \text{ ml}$  of ethyl acetate. After centrifugation, an aliquot ( $1.5 \text{ ml}$ ) of the organic phase was evaporated to dryness under a stream of nitrogen in a waterbath at  $40^\circ\text{C}$ . The residue was silylated (tBDMS, see below) and analyzed by GC-MS (selected ion detection mode) on the ion at  $m/z$  481 for all three compounds.

#### *Determination of the extraction efficiency*

Methyl formate derivatives of catecholamines (E, NE and IP,  $1 \mu\text{g}$  of each) were extracted into ethyl acetate at a phase ratio of 1. Then  $1 \text{ ml}$  of the water phase was transferred to a clean tube and re-extracted with  $1 \text{ ml}$  of ethyl acetate. An aliquot of both organic layers ( $0.5 \text{ ml}$ ) was evaporated to dryness and silylated (tBDMS). The amounts of catecholamines were estimated by GC relative to octacosane as internal standard ( $5 \mu\text{g}$  first extract,  $250 \text{ ng}$  second extract).

#### *Evaluation of silylation conditions*

Methyl formate derivatives of catecholamines were treated with various silylating reagents under various conditions.

Identification of the derivatives formed was performed by GC-MS. The GC oven temperature was  $240^\circ\text{C}$  and  $270^\circ\text{C}$  for TMS and tBDMS derivatives, respectively. The following silylating methods were tested: (1)  $100 \mu\text{l}$  of HMDS + 1% TMCS (pure or 20% in dichloromethane) for 5, 30 and 90 min at  $20^\circ\text{C}$ , and for 5, 50 and 240 min at  $70^\circ\text{C}$ ; (2)  $100 \mu\text{l}$  of BSTFA + 1% TMCS (pure and 10% in acetonitrile) for 5, 30, 90 min and overnight at  $20^\circ\text{C}$ , and for 5, 50 and 240 min at  $70^\circ\text{C}$ ; (3)  $100 \mu\text{l}$  of TSIM (5% in diethyl ether or dichloromethane) for 5, 30 and 90 min at  $20^\circ\text{C}$ ; (4)  $100 \mu\text{l}$  of tBDMCS-imidazole-dimethylformamide for 90 min at 60, 80 and  $110^\circ\text{C}$ , after cooling the reaction mixture to room temperature,  $100 \mu\text{l}$  of hexane were added to dissolve the derivatives.

#### *Calibration curves*

Unhydrolyzed urine ( $0.25 \text{ ml}$ ) was spiked with epinephrine (range  $0\text{--}50 \text{ ng}$ ), norepinephrine (range  $0\text{--}170 \text{ ng}$ ) and isoprenaline (internal standard,  $51.08 \text{ ng}$  in  $0.2 \text{ ml}$  of  $0.02 \text{ N}$  hydrochloric acid) and processed as given in Fig. 1.

#### *Biological samples*

*Urine.* Catecholamine conjugates in urine were hydrolyzed by boiling acidified urine ( $\text{HCl}$ ,  $\text{pH} \leq 1$ ) for  $20 \text{ min}$ . To an aliquot of  $0.5 \text{ ml}$  were added  $51.08 \text{ ng}$  of isoprenaline (internal standard),  $25 \mu\text{l}$  of methyl chloroformate and

2.0 ml of phosphate buffer (pH 7.5). The pH shift was made with 1.0 ml of the carbonate buffer (pH 11). For other conditions see Fig. 1.

*Plasma.* To 0.5 ml of non-preprocessed plasma were added 1.27 ng of isoprenaline (internal standard). The emulsion, which occasionally occurs after extraction, was broken by stirring with a small glass rod and the sample was centrifuged again.

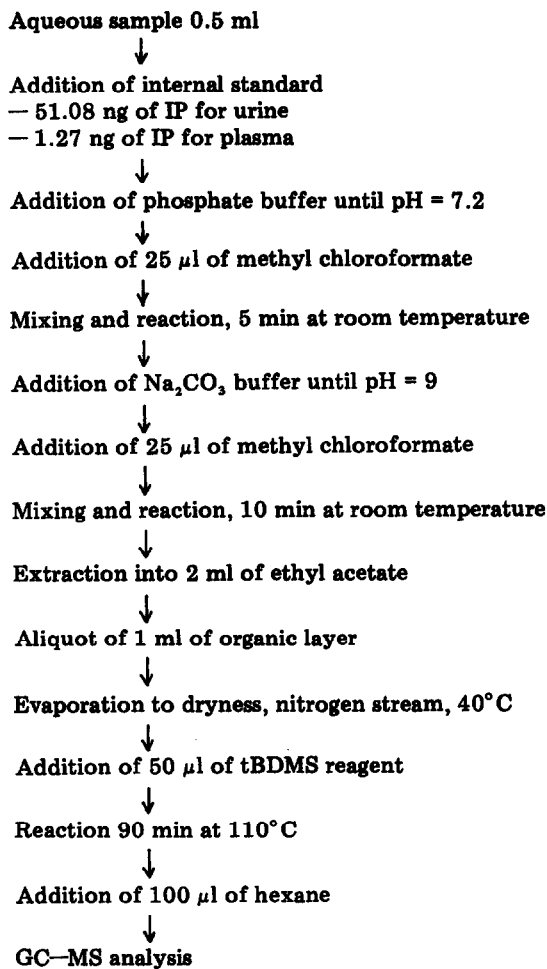


Fig. 1. Flow chart of analytical procedure for determination of catecholamines and metanephrines in biological samples.

## RESULTS

### *pH of aqueous reaction medium*

Fig. 2 shows the yield of the reaction of methyl chloroformate with 1,2-dihydroxybenzene as a function of the pH of the aqueous medium. The

maximum yield is reached at pH 7.2. The reaction takes place virtually instantaneously. Maximum yield was obtained for a reaction time of 1 min at pH 7.2. For practical reasons, e.g. reproducibility of sample handling of large series, the reaction conditions have been fixed at a pH of 7.2 and a reaction time of 5 min. In order to estimate simultaneously the reaction with primary amino groups, these conditions were applied to dopamine. No other conditions have been found which resulted in higher yields of the N,O-tricarbamate derivative. A linear relationship between the amount of dopamine and the yield of the derivative was observed over the range studied:  $Y = 0.753X - 0.015$ ;  $r = 0.9997$ , range 15–150  $\mu\text{g}$ .

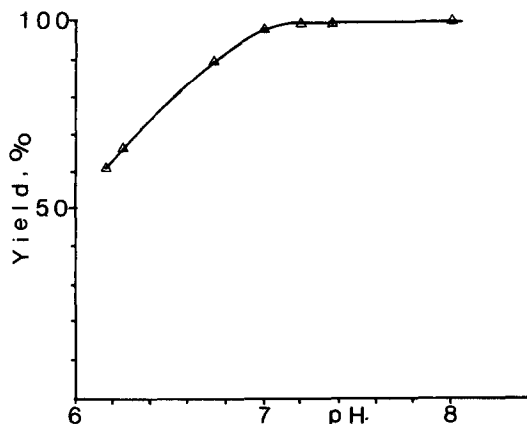


Fig. 2. Yield of the methylformate derivative of 1,2-dihydroxybenzene relative to  $n\text{-C}_{14}$ , as a function of the pH of the aqueous medium. Reaction 30 min at room temperature.

For secondary amines, in particular for isoprenaline, lower yields and less reproducible results were obtained. Both the yield and the reproducibility were considerably improved by a pH shift of  $\Delta\text{pH} = 2$  after incubation of the reaction mixture for 5 min at pH 7.2. The effect of the pH shift on the yield of the formate derivative of isoprenaline and epinephrine relative to norepinephrine is shown in Fig. 3.

#### Isolation of formate derivatives

The extraction recoveries of epinephrine, norepinephrine and isoprenaline were determined for the system ethyl acetate–aqueous medium as 99.65%, 99.50% and 99.83% using a phase ratio of 1. The calculated partition constants are 290, 200 and 605, for E, NE and IP, respectively.

#### Silylation

In our hands, silylation of formate derivatives of catecholamines with HMDS [11] yielded mixed derivatives of form II and III together with minor amounts of form IV (cf. Fig. 4). Even under milder conditions (e.g. 10% HMDS in ethyl acetate) no single peak of form II could be obtained. Neither with stronger

silylating reagents nor under vigorous conditions has a single derivative been obtained.

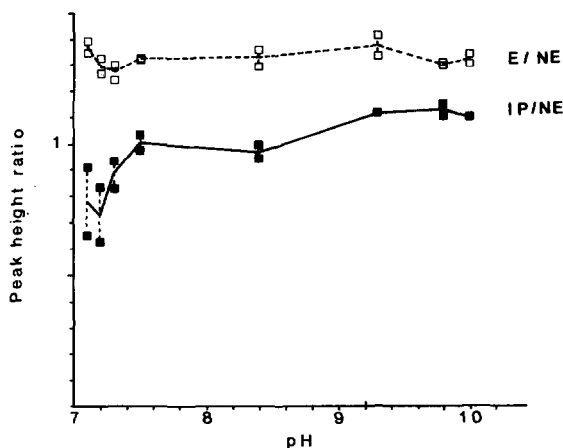


Fig. 3. Influence of the pH shift step on the yield and reproducibility for the reaction of methyl chloroformate with N-alkyl-substituted catecholamines. E = epinephrine, IP = isoprenaline and NE = norepinephrine (internal standard).

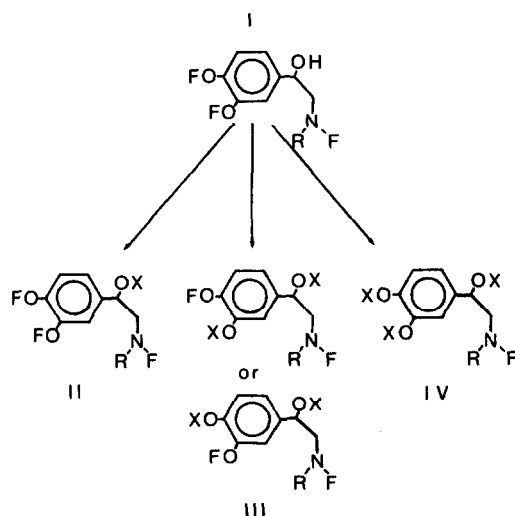


Fig. 4. Trialkylsilyl derivative formation of methyl formate derivatives of catecholamines. F =  $\text{CH}_2\text{O}-\text{CO}$ ; X =  $(\text{alkyl})_3-\text{Si}$ ; R = H,  $\text{CH}_3$ , or  $\text{CH}(\text{CH}_3)_2$ . I = Catecholamine-N,O,O-tris-methyl formate derivative. II, III and IV: No, partial and complete exchange of the O-methyl formate groups with silyl groups, respectively.

If the reaction was enforced too strongly, partial N-silylation took place for primary amines (NE, NMN and DA). This has been avoided by using TSIM, a very weak silyl donor for aliphatic amino groups [14]. A 5% mixture of TSIM in dichloromethane or diethyl ether yielded a single peak of form IV, but the yield was not very reproducible. Although the tBDMCS-imidazole reagent is less reactive than most of the TMS reagents [15], it is able to exchange O-

TMS with O-tBDMS [16]. It appears that this ability also includes O-carbamate groups. Substitution of O-carbamate together with silylation of the aliphatic hydroxy group was completed within 90 min at 110°C.

#### Gas chromatography—mass spectrometry

Fig. 5 shows a mass chromatogram of a synthetic mixture of metanephrines (MN and NMN) and catecholamines (E, NE and IP) as their O-tBDMS, N-carbamate derivatives. Up to 270°C a baseline separation was achieved on a medium polar capillary column (OV-1701). On non-polar capillary columns (SE-30) both the metanephrines (MN and NMN) and the catecholamines (E and NE) eluted together.

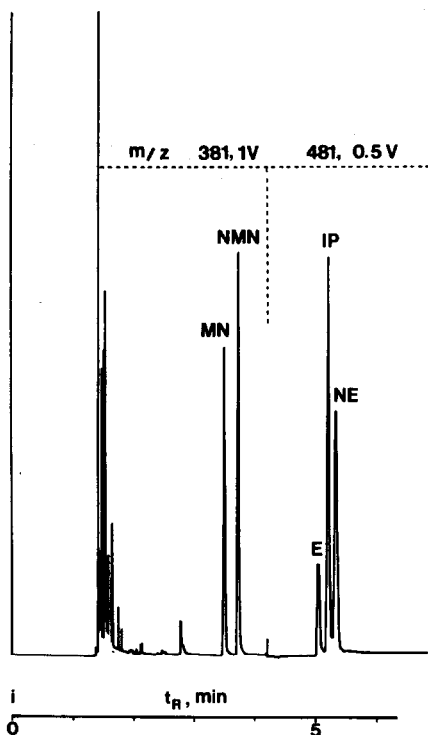


Fig. 5. Mass chromatogram of a synthetic mixture of metanephrines and catecholamines. MN = metanephrine (20 ng), NMN = normetanephrine (40 ng), E = epinephrine (6 ng), IP = isoprenaline (51 ng) and NE = norepinephrine (21 ng).

The retention indices of these compounds are given in Table I. The mass spectra of the compounds of interest are dominated by  $\alpha$ -cleavage of the hydroxyethylamino chain, resulting in very intense ions at mass 381 and 481 for metanephrines and catecholamines, respectively. The mass spectral data of the catecholamines are summarized in Table II.



TABLE I

## RETENTION INDICES ON OV-1701 OF O-tBDMS, N-CARBAMATE DERIVATIVES OF CATECHOLAMINES

Compound	Retention index
Metanephrine	2696
Normetanephrine	2733
Epinephrine	2908
Isoprenaline	2924
Norepinephrine	2937

TABLE II

## MASS SPECTRAL DATA OF O-tBDMS, N-CARBAMATE DERIVATIVES OF CATECHOLAMINES

Compound	Fragment a* % $\Sigma_{70} I^{**}$	Base peak <i>m/z</i>	Other peaks <i>m/z</i> (relative intensity)
Epinephrine	12.2	481	73 (73), 75 (18), 208 (8), 526 (3)
Norepinephrine	9.1	73	481 (44), 75 (38), 77 (19), 480 (5)
Isoprenaline	15.3	481	73 (93), 75 (22), 77 (20), 554 (2)

\*Fragment of  $\alpha$ -cleavage of hydroxyethylamino chain.

\*\*%  $\Sigma_{70} I$  = Intensity relative to the total ionization in the mass range *m/z* 70 to the molecular ion.

*Quantitative results*

The linearity of the whole procedure (carbamate formation, extraction, silylation and GC-MS analysis) was determined for physiological ranges of E and NE in urine. These experiments were performed in diluted hydrochloric acid and in spiked unhydrolyzed urine. Apart from the vertical displacement, due to the presence of free endogenous catecholamines in the urine, both calibration curves were identical when expressed in terms of slope, coefficient of regression and coefficient of variation. The calibration curves for the determination of the catecholamines in urine were linear over the range studied. The equations for E and NE were as follows:

$$E: Y = 1.31X + 0.01, r = 0.9995, \text{ range } 3\text{--}50 \text{ ng}$$

$$NE: Y = 1.105X + 0.058, r = 0.9993, \text{ range } 9\text{--}150 \text{ ng}$$

where *Y* is the ratio of the peak heights of the catecholamine and the internal standard and *X* is the ratio of the amounts (w/w) of the catecholamine and the internal standard.

The precision of the procedure was determined from a set of six identically processed aliquots of a hydrolyzed urine. The coefficients of variation were 5.5% and 1.6% for E and NE, respectively, for a mean sample concentration of 9.7 ng ml<sup>-1</sup> of E and 74.4 ng ml<sup>-1</sup> of NE. The analytical recovery was estimated by adding of 7.47 ng of E and 34.84 ng of NE to an aliquot of 0.5 ml of this urine. Recovered were 7.24 ng of E (97%) and 33.1 ng of NE (95%).

The detection limit of the GC-MS was 5 pg with a signal-to-noise ratio of 3.

This sensitivity allowed the determination of catecholamines down to  $50 \text{ pg ml}^{-1}$ . The determination of catecholamines in normal plasma samples has not yet been evaluated.

### Applications

Fig. 6 shows an example of the analysis of metanephrines and catecholamines in a normal urine. An example of an assay in plasma of a patient suffering from pheochromocytoma is given in Fig. 7.

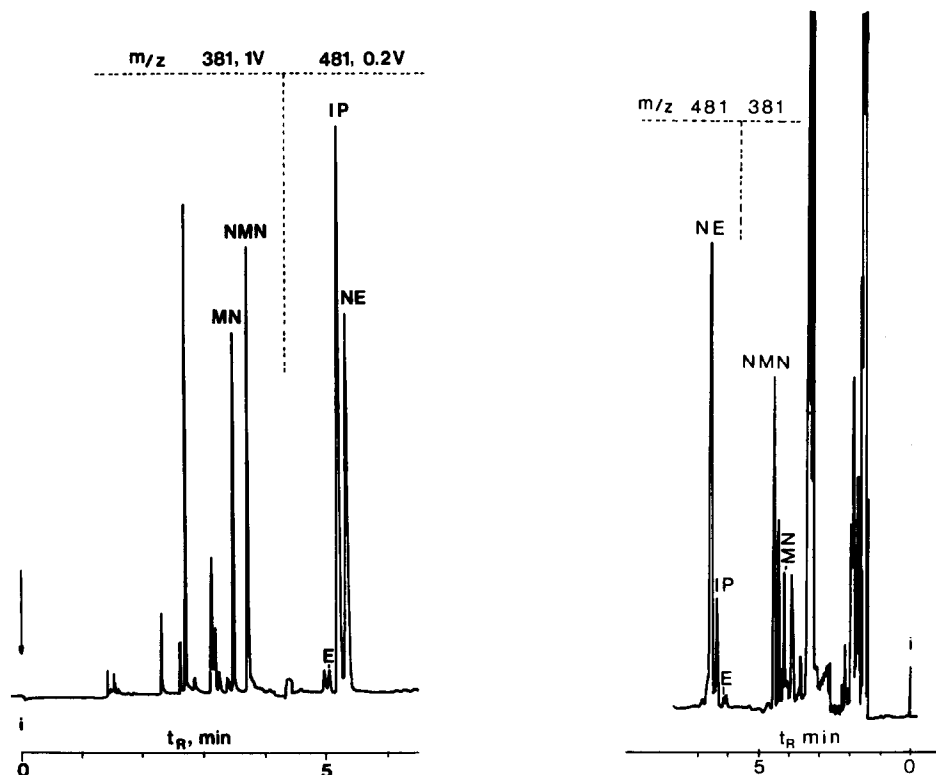


Fig. 6. Analysis of metanephrines and catecholamines in a normal urine. MN = metanephrine ( $1.15 \mu\text{mol}$  per 24 h), NMN = normetanephrine ( $2.25 \mu\text{mol}$  per 24 h), E = epinephrine ( $0.03 \mu\text{mol}$  per 24 h) and NE = norepinephrine ( $0.80 \mu\text{mol}$  per 24 h).

Fig. 7. Analysis of metanephrines and catecholamines in plasma of a patient suffering from pheochromocytoma. Amount of internal standard (IP) =  $1.27 \text{ ng}$ .

### DISCUSSION

The kinetics of the reaction of alkyl chloroformates with amines in buffered aqueous media have been described by Ahnfelt and Hartvig [12]. The kinetic model for the reaction





is given by

$$\ln \frac{C_0^{\text{AH}}}{C_t^{\text{AH}}} = \frac{k_1 \cdot C_t^{\text{F}} \cdot K_a}{k_s \cdot (K_a + a_{\text{H}^+})}$$

with  $C_t^{\text{F}} = C_0^{\text{F}} (1 - e^{-k_1 t}) \sim C_0^{\text{F}}$  for  $1 \gg e^{-k_1 t}$  where

$C_t^{\text{AH}}$  = concentration of the amine at time  $t$  (M)

$C_t^{\text{F}}$  = concentration of chloroformate at time  $t$  (M)

$k_1$  = rate constant for the reaction alkyl chloroformate with the amine ( $\text{mol}^{-1} \text{sec}^{-1}$ )

$k_s$  = hydrolysis rate constant of the chloroformate ( $\text{sec}^{-1}$ )

$K_a$  = acid dissociation constant of the amine

$a_{\text{H}^+}$  = activity of  $\text{H}_3\text{O}^+$ .

Although this equation is not valid for multifunctional compounds, consideration of the variables will be of great help in solving kinetic problems. In the present case, isoprenaline appeared to be less reactive than norepinephrine. This can partially be ascribed to the difference between the  $K_a$  of the amine functions of NE ( $\text{p}K_a = 9.78$ ) and IP ( $\text{p}K_a = 10.0$ ). More important for the lower reactivity of isoprenaline is probably the steric hindrance caused by the N-isopropyl group (decrease of  $k_1$ ). Both negative effects on the yield can effectively be compensated by increasing the pH, so that the right-hand side of eqn. 1 yields a higher numerical value ( $> 4.6$  for  $> 99\%$  conversion). The observation of lower reactivity of compounds with a bulky N-alkyl substituent is in good agreement with the findings of Gyllenhaal et al. [11] on the carbamate formation of *n*-hexylnorepinephrine. However, increasing the pH is not allowed until the catecholic function has been protected. At elevated pH a considerable amount of the catecholamines would be oxidized before the carbamate formation was completed. The fact that the phenolic hydroxy groups react rapidly with methyl chloroformate at a relatively low pH permits the use of a pH shift.

The formation of tBDMS derivatives has substantial advantages above TMS derivatives: (1) the derivatives are much more stable [15]; (2) the increased mass and the higher intensity of the analyzed ion improves the signal-to-noise ratio in both ways; (3) the substitution of O-carbamate by tBDMS while the N-carbamate group is not affected provides a powerful tool to increase the selectivity. This implies that only amines will retain the carbamate moiety. Consequently, only amines will be specifically detected if a specific alkyl chloroformate has been used. A typical example would be 2,2,2-trichloroethyl chloroformate as reagent and GC with electron-capture detection or GC-MS with electron attachment negative chemical ionization. These mixed derivatives will have comparable properties to the O-TMS, pentafluorobenzylimide derivative of catecholamines as described by Lhuguenot and Maume [17]. However, these imide derivatives can only be formed with primary amines, whereas the derivatives as described here can be formed with both primary and secondary amines.

In conclusion, the derivatization of catecholamines and related compounds (e.g. their synthetic congeners) in aqueous biological samples provides a rapid,

easy and accurate isolation method with a high and reproducible yield. The O-tBDMS, N-carbamate derivative formation provides an excellent method to investigate in a very selective and sensitive way the occurrence of catecholamines in biological fluids. This method therefore represents a meaningful procedure next to the frequently used radioenzymatic [18] and liquid chromatography with electrochemical detection [19] methods.

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