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DERIVATIZATION AND MASS SPECTROMETRIC BEHAVIOUR OF CATECHOLAMINES AND THEIR 3-O-METHYLATED METABOLITES

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SUMMARY

A method has been developed for the derivatization of both catecholamines (dopamine, noradrenaline and adrenaline) and their 3-O-methylated metabolites (3-methoxytyramine, normetanephrine and metanephrine) in a single run. The compounds were first incubated with methanolic hydrochloric acid to methylate those compounds that contain a benzylic hydroxyl group and were subsequently converted into their pentafluoropropionyl derivatives. The derivatives thus prepared, showed good gas chromatographic and electron-impact mass spectrometric properties and can be analysed in a single gas chromatographic run.

The effect of the derivatization on exchange reactions in the aromatic ring was investigated because standard compounds with deuterium label in that part of the molecule are often used in isotope dilution measurements. The exchange of deuterium for hydrogen in the aromatic ring under derivatization conditions was found to be limited.

INTRODUCTION

In recent years a variety of analytical methods has been developed for the determination of catecholamines and their 3-O-methylated metabolites [1—7]. Among these methods capillary gas chromatography—multi ion detection mass spectrometry (GC—MID-MS) affords a combination of great selectivity and sensitivity which is not easily obtained by other methods. Moreover, a high accuracy can be obtained with the isotope dilution method if stable isotopically labelled compounds are available.

GC—MS can be used for routine quantitative measurements and for confirmation of the results obtained with other methods. The fact that several compounds can be measured in a single run makes the method appropriate for profiling purposes. Such applications actually require only one derivatization method for a group of related compounds to afford suitable derivatives for a particular ionization method.

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Very recently such a derivatization method has been reported by De Jong and Cramers [8] which enables the extraction of catecholamines and their 3-O-methylated metabolites via the formation of their formyl esters, followed by an additional conversion with *tert.*-butyldimethylsilyl chloride to O-*tert.*-butyldimethylsilyl, N-formate derivatives. In this way profiles are obtained from these compounds, except from the more volatile 3-methoxytyramine.

In this paper a derivatization method is described for converting in one run of dopamine, noradrenaline, adrenaline, 3-methoxytyramine, normetanephrine and metanephrine into derivatives with good electron-impact properties. It appeared that after this derivatization six compounds can easily be recorded in a single profile.

In many quantitative GC—MID-MS applications deuterium-labelled compounds are used for isotope dilution. With the catecholamines and their 3-O-methylated metabolites deuterium labelling is often positioned on the aromatic ring. In this connection, the stability of the compounds under derivatization conditions with respect to exchange reactions in the aromatic ring was investigated too.

EXPERIMENTAL

Reagents and materials

Analytical grade methanol, deuteromethanol, acetyl chloride and ethyl acetate were obtained from E. Merck (Darmstadt, F.R.G.) and pentafluoropropionic anhydride from Pierce (Rockford, IL, U.S.A.). DL-3,4-dihydroxyphenylalanine, 3-methoxytyrosine, dopamine, adrenaline, noradrenaline, 3-methoxytyramine, metanephrine and normetanephrine were purchased from Sigma (St. Louis, MO, U.S.A.). The compounds were dissolved in methanol (0.5 g/l) and stored at −15°C for a period not exceeding one month.

Solutions of hydrochloric acid in methanol were prepared by addition of acetyl chloride to methanol. A 1 *M* ^{2}HCl solution in deuteromethanol was prepared by addition of acetyl chloride to deuteromethanol.

The derivatization procedure was carried out in screw-capped tubes (7 ml, Sovirel 461151 P).

Derivatization

In order to find the optimal conditions for the synthesis of the benzylic-O-methyl (β-MeO) compounds of noradrenaline (NA), adrenaline (A), normetanephrine (NM) and metanephrine (M), the reaction with methanol–hydrochloric acid was carried out at three different reaction times (0.5, 1 and 4 h) and three hydrochloric acid concentrations (0.5, 1.1 and 2.1 *M*) at room temperature. An additional experiment was carried out, using 1.1 *M* hydrochloric acid at 60°C for 1 h. Although dopamine (DA) and 3-methoxytyramine (3-MT) do not possess a benzylic hydroxyl group, these compounds were also included in the first derivatization step. Yields were estimated from the peak heights in the reconstructed total ion current (RTIC) chromatogram.

A 0.1 ml volume of a methanol solution of catecholamines and 3-O-methylated catecholamines (0.5 g/l) was evaporated to dryness in a stream of nitrogen. A volume of 0.1 ml methanol—hydrochloric acid was added and

the sample was allowed to stand at room temperature. Subsequently, the methanol–hydrochloric acid was evaporated in a stream of dry nitrogen. A 0.1 ml volume of pentafluoropropionic anhydride (PFPA) was added and the sample was heated at 80°C for 15 min. After cooling to 20–30°C the excess reagent was evaporated in a gentle stream of dry nitrogen. A 0.1 ml portion of ethyl acetate containing 4% (v/v) PFPA was added to dissolve the derivatives.

Exchange reactions were carried out by incubating the compounds with 0.1 ml of 1 *M* deuteromethanol—^{2}HCl at room temperature for 1 h.

Gas chromatography–mass spectrometry

A Varian gas chromatograph 3700 coupled by an open split interface [9] to a Finnigan-MAT 212 mass spectrometer was used. The gas chromatograph was equipped with a capillary column (CP Sil 5, 45 m × 0.5 mm I.D.; Chrompack, Middelburg, The Netherlands). Helium (5 ml/min) was used as carrier gas. The column temperature was set at 170°C. The GC injection port and the interface region were maintained at 225°C and 220°C, respectively; the line-of-site temperature was kept at 200°C. The mass spectrometer was operated at an electron energy of 70 eV and an emission current of 1 mA. The ion source temperature was maintained at 220°C. For the identification of the derivatized compounds, spectra were taken in the mass range 23–800 with a scan speed of 1 decade/sec. Data were collected and stored in a Finnigan MAT SS200 computer system.

To investigate the exchange reaction of deuterium for hydrogen, the fragment ion + 1/fragment ion ratios of the derivatives were measured with MID under computer control. These ratios were compared with those from the same derivatives, prepared at the same time with methanol–hydrochloric acid under similar conditions. DA was measured at *m/z* 429/428; NA and A at *m/z* 446/445; 3-MT at *m/z* 297/296; NM and M at *m/z* 314/313.

RESULTS AND DISCUSSION

Optimization of the benzylic-O-methylation

During quality control it was observed that the hydrochlorides of NA, A, NM and M standard compounds dissolved in methanol and stored at 4°C were slowly converted. When the standard solution was stored under this condition for two to four months, all these compounds showed a satelite peak in the gas chromatogram after derivatization with PFPA. Such a conversion was not observed with DA and 3-MT, which indicates that the benzylic hydroxyl group was involved. The satelite peaks were identified by mass spectrometry as the β-MeO-PFP compounds (compounds 4, 6, 9 and 10, Fig. 1).

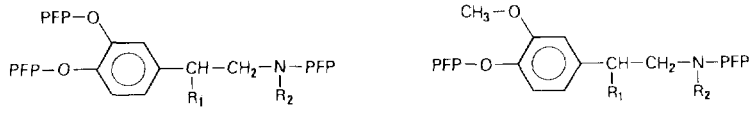

Fig. 1. Derivatives of catecholamines and their 3-O-methylated metabolites: 1 = NA (R_1 = OPFP; R_2 = H); 2 = A (R_1 = OPFP; R_2 = CH_3); 3 = DA (R_1 = H; R_2 = H); 4 = NA (R_1 = OCH_3; R_2 = H); 5 = NM (R_1 = OPFP; R_2 = H); 6 = A (R_1 = OCH_3; R_2 = CH_3); 7 = 3-MT (R_1 = H; R_2 = H); 8 = M (R_1 = OPFP; R_2 = CH_3); 9 = NM (R_1 = OCH_3; R_2 = H); 10 = M (R_1 = OCH_3; R_2 = CH_3).

Compounds 4 and 6 were recently prepared by Martin et al. [10], who studied GC and mass spectral characteristics of these compounds. Arnold and Ford [11] reported the analysis of compounds 9 and 10 in brain tissue, using GC with electron-capture detection. Also the corresponding benzylic-O-ethyl derivatives were applied in a GC—MS assay of human plasma catecholamines [4].

From the literature and our own data it was concluded that the preparation of the β-MeO-PFP compounds would be a useful method for combined derivatization of catecholamines and their 3-O-methylated metabolites.

By varying the incubation time and the hydrochloric acid concentration, the benzylic-O-methylation was found to have a near optimum when the reaction was carried out for 1 h in 1 *M* methanol—hydrochloric acid at 20°C (Table I). Extended reaction times and increased hydrochloric acid concentrations gave slightly higher yields, but on the other hand resulted in some by-product formation. These by-products were identified as the β-chloroethyl analogues of NA and A (R_1 = Cl, Fig. 1). Application of a reaction temperature of 60°C resulted in very low yields, probably caused by an increased β-chloroethyl formation followed by a further decomposition. With none of the compounds could a complete conversion be achieved. Therefore, after reaction with PFPA the "all"-PFP derivatives 1, 2, 5 and 8 were also observed in the RTIC chromatogram (Figs. 1 and 2).

TABLE I

OPTIMIZATION OF THE BENZYLIC-O-METHYLATION OF NA, A, NM AND M

Reaction at room temperature of NA, A, NM and M (50 μg in 100 μl of methanol) at different hydrochloric acid concentrations and reaction times. Yields were estimated by means of the peak heights in the reconstructed total ion current chromatogram after derivatization with PFPA.

HCl concentration (*M*)	Reaction time (h)	Yield (%)			
		NA	A	NM	M
0.5	1	56	47	34	32
1.1	0.5	71	66	40	35
1.1	1	90	75	60	46
1.1	4	93	89	84	67
2.1	1	91	88	63	57

Despite the fact that DA and 3-MT do not react during the β-O-methylation step, the compounds can be included in the first derivatization step, yielding PFP derivatives in the subsequent reaction with PFPA, thus enabling derivatization of six compounds in a single run.

Under the GC conditions applied, all derivatives were well separated. The β-MeO-PFP derivatives showed longer retention times than the corresponding "all"-PFP derivatives. When dissolved in ethyl acetate—4% (v/v) PFPA the derivatives were stable for at least 24 h at ca. 4°C.

Some experiments were carried out to ascertain whether the same derivatization method could be applied to convert 3,4-dihydroxyphenylalanine and 3-methoxytyrosine into their methyl ester PFP derivatives, as was reported by Arnold and Ford [11]. However, under the conditions applied these attempts

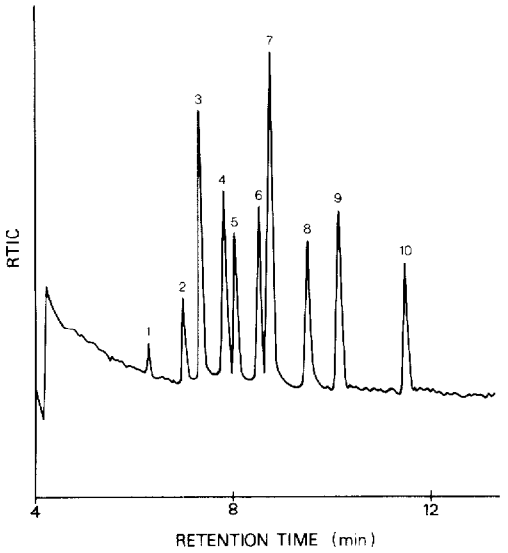

Fig. 2. Reconstructed total ion current (RTIC) chromatogram of a derivatized mixture of catecholamines and 3-O-methylated catecholamines. GC column and conditions are given in the experimental section. Peak numbers refer to the compounds mentioned in the legend to Fig. 1.

remained unsuccessful, probably because the esterification step only resulted in low yields.

Mass spectral characteristics

The sensitivity and selectivity of GC—MID-MS assays depend on the presence of high-intensity fragments or molecular ions in the higher mass region. The β-MeO-PFP derivatives of NA, A, NM and M (compounds 4, 6, 9 and 10, Fig. 1) show very dominant ring-containing fragments at *m/z* 445 (NA and A) and at *m/z* 313 (NM and M), which make them suitable for MID under electron-impact conditions. These fragments result from cleavage of the side-chain between the α- and the β-position, with charge retention on the aromatic part of the molecule. As shown in Table II, these fragments account for a considerable part of the total ion current. The excellent mass spectrometric properties of this type of derivative of NM and M have already been described by Martin et al. [10].

The PFP derivatives of DA and 3-MT (compounds 3 and 7, Fig. 1) have their base peaks at *m/z* 428 and *m/z* 296, respectively. Both fragments are formed via a McLafferty rearrangement and contain the aromatic ring and the α,β part of the side-chain [12]. In this case ring-deuterated as well as side-chain-deuterated standard compounds can be used for isotope dilution measurements.

The "all"-PFP derivatives of NA, A, NM and M (compounds 1, 2, 5 and 8, Fig. 1) show less favourable fragmentation characteristics. NA and A possess ring-containing fragments of low intensity at *m/z* 590 and *m/z* 604, respectively. The less specific side-chain fragments at *m/z* 176 and *m/z* 190 dominate. With NM and M the ring-containing fragment at *m/z* 458 is of medium intensity.

Comparison of the β-MeO-PFP derivatives with the "all"-PFP derivatives

TABLE II

MASSES $m/z > 170$ AS PERCENTAGE OF THE BASEPEAK INTENSITY (B) AND OF THE TOTAL ION INTENSITY (I), BOTH FROM THE MASS RANGE m/z 41—800

	Mass	B	I		Mass	B	I
1. NA	176	21	9	2. A	190	100	40
	590	<1	<1		191	4	2
					604	<1	<1
3. DA	176	54	11	4. NA	176	2	1
	177	3	1		179	5	2
	225	5	1		267	2	1
	253	3	1		299	3	1
	265	8	2		417	2	1
	269	3	1		445	100	37
	281	39	8		446	12	4
	282	3	1		447	3	1
	387	6	1		458	3	1
	428	100	21				
	429	22	5				
5. NM	176	7	1	6. A	179	9	2
	270	6	1		190	24	7
	298	5	1		191	2	1
	311	8	2		267	3	1
	417	7	1		417	2	1
	445	11	2		445	100	28
	458	27	5		446	26	7
	459	5	1		447	3	1
					472	3	1
7. 3-MT	176	4	1	8. M	190	100	35
	255	4	1		191	3	1
	283	19	5		311	2	1
	284	3	1		445	3	1
	296	100	26		458	18	6
	297	16	4		459	3	1
	298	2	1		471	2	1
	459	6	2		472	2	1
9. NM	298	2	1	10. M	190	4	2
	313	100	41		313	100	42
	314	14	6		314	10	4

shows that the former group of derivatives has better mass spectrometric properties, because the ring-containing fragments in the higher mass region are more intense.

Exchange reaction of deuterium for hydrogen in the aromatic ring

Because the robust ^{13}C-labelled compounds are often not commercially available at the required degree of labelling, or not available at all, deuterium-labelled compounds are more frequently used.

With catecholamines and 3-O-methylated catecholamines, deuterium labelling is often carried out in the aromatic ring. Only recently a method has been developed for the synthesis of 3-O-deuteromethylated catecholamines

[13]. These compounds have a good stability under acidic conditions. Although side-chain-deuterated NM and M are commercially available now, their use is limited to those ionization methods that do not split off the side-chain as a fragment. Therefore, we were interested in the stability of compounds with respect to exchange reactions in the aromatic ring.

In this connection, measurements were carried out of the ratios of the fragment ion + 1/fragment ion of derivatives prepared in deuteromethanol—^{2}HCl, and those of derivatives prepared in methanol—hydrochloric acid (Table III). These ratios indicate that NA, A, NM, M and 3-MT show no or hardly any exchange. The loss of ring deuterium label for NM and M, as was observed by Martin et al. [10], was not confirmed in our experiments. With DA a slightly higher exchange rate was observed; the 429/428 ratio was ca. 2.3% higher. When exact measurements are needed, the use of side-chain-labelled DA is to be preferred.

TABLE III

INFLUENCE OF THE DERIVATIZATION ON THE FRAGMENT ION + 1/FRAGMENT ION RATIOS

Reaction at room temperature of catecholamines and 3-O-methylated catecholamines, 50 μg in 100 μl of 1 *M* deuteromethanol—^{2}HCl and 100 μl of 1 *M* methanol—hydrochloric acid, respectively, for 1 h. The fragment ion + 1/fragment ion ratios are given in %. NA and A were measured at *m/z* 446/445; DA at *m/z* 429/428; NM and M at *m/z* 314/313; 3-MT at *m/z* 297/296. The figures given are the mean values ($\bar{x}$) and the standard deviations (S.D.) of eight replicate reactions.

	Deuteromethanol—^{2}HCl		Methanol—HCl		$\Delta\bar{x}$
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	
NA	15.4	0.4	15.1	0.4	+0.3
A	15.3	0.6	15.1	0.1	+0.2
DA	23.0	3.2	20.7	1.1	+2.3
NM	12.6	0.3	12.7	0.3	−0.1
M	12.4	0.5	12.8	0.3	−0.4
3-MT	17.3	0.9	16.7	0.3	+0.6

Our experiments, carried out with unlabelled standard compounds, give an overestimation of the exchange rate of the labelled compounds, since deuterium-labelled compounds are less susceptible to exchange reaction. This isotope effect is caused by the higher activation energy needed for the cleavage of a carbon- deuterium bond [14].

CONCLUSION

A simple derivatization method has been developed for the combined conversion of catecholamines and their 3-O-methylated metabolites. The exchange of deuterium for hydrogen in the aromatic ring was found to occur only to a limited extent.

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