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DIRECT DERIVATIZATION OF ALPRENOLOL AND ITS 4-HYDROXY METABOLITE IN URINE WITH PHOSGENE AND METHANOL PRIOR TO ANALYSIS BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

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SUMMARY

A method for the determination of alprenolol and its 4-hydroxy metabolite has been developed. The urine sample is made alkaline with buffer (pH 12) and derivatized with 60 μl of 2 *M* phosgene in toluene with vigorous shaking. In the presence of 2.5% methanol, an oxazolidineone methyl carbonate is formed from 4-hydroxy alprenolol. The now neutral derivatives are extracted with an equal volume of dichloromethane. After evaporation of the organic phase, the residue is taken up in a small volume of ethyl acetate and subjected to capillary column gas chromatography with CP-Sil 8 as the stationary phase. The precision was 2.1% at the 3.3 μg/ml level of the metabolite in urine (n = 8). The isopentylamino analogue was used as the internal standard.

INTRODUCTION

Alprenolol, 1-(2-allylphenoxy)-3-isopropylamino-2-propanol, is an unselective β-adrenoreceptor antagonist that has been used clinically since 1967. Its major metabolite is 4-hydroxy alprenolol[1,2], which possesses approximately the same activity as the parent drug[3].

Aminoalcohols can be derivatized to the corresponding oxazolidineone derivatives. This reaction was the basis for analysis of the β-blocker metoprolol[4] and its major and minor metabolites[5,6] in urine by gas chromatography. Also, metoprolol base can be used as a reagent for the determination of phosgene in dichloromethane formed in the presence of light[7]. This type of derivative has also been used for the chiral resolution of enantiomers by capillary column gas chromatography[8–9] and by liquid chromatography[10–12].

Not only can 2-, 3- and 4-amino alcohols[4] be derivatized, but also glycols and 2-hydroxyacids as well as N-methylamino acids[13]. Lone hydroxyl or carboxyl groups do not react with phosgene but must be silylated before gas chromatography[5,6].

However, the cyclization reaction with phosgene might be complicated by the presence of other functional groups that are attacked by phosgene, such as amines and phenols. An amine related to metoprolol could be gas-chromatographed as its chlorocarbonyl derivative[4]. However, at low sample levels, conversion to its methyl

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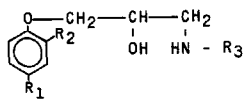
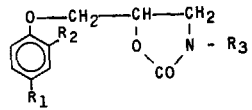
TABLE I

STRUCTURES OF COMPOUNDS STUDIED AND DERIVATIVES FORMED

Before derivatization



After derivatization



Compound	R_1	R_2	R_3	R_1, *after derivatization*	*Type of derivative*
Alprenolol	–H	$-CH_2-CH=CH_2$	$-CH(CH_3)_2$	–H	Oxazolidine-2-one
H 56/60 (internal standard)	–H	$-CH(CH_3)_2$	$-CH(CH_3)_2$	–H	Oxazolidine-2-one
4-Hydroxy alprenolol	–OH	$-CH_2-CH=CH_2$	$-CH(CH_3)_2$	$-OCOOCH_3$	Oxazolidine-2-one 4-methyl carbonate
H 155/48 (internal standard)	–OH	$-CH_2-CH=CH_2$	$-CH(C_2H_5)_2$	$-OCOOCH_3$	Oxazolidine-2-one 4-methyl carbonate

carbamate with methanol was a prerequisite for proper chromatographic behaviour[4].

The present investigation was undertaken in order to improve the understanding of, and to extend the usefulness of, phosgene as a derivatizing reagent for multifunctional compounds. Interest was focused on 4-hydroxy alprenolol.

EXPERIMENTAL

Gas chromatography

A Varian 3700 gas chromatograph was used, equipped with a flame-ionization and a nitrogen-selective detector. The capillary column was connected to the injector with adaptors to allow split or splitless injections and to introduce make-up gas into the detector. The injector liner was silanized and filled with a *ca.* 1-cm long quartz-wool plug between the column end and the syringe tip/needle. The inlet pressure of the nitrogen carrier gas was 100 kPa, giving a flow-rate of 60 cm/s. The injector and the detector were both maintained at 300°C. The oven temperature was kept at 100°C for 1 min after the injection and then increased at a rate of 20°/min to 280°C. The fused-silica column used (22 m × 0.32 mm I.D.) was coated with CP-Sil 8 from Chrompack (Middelburg, The Netherlands). Injections were made under splitless conditions. The vent valve was opened after 1.5 min and closed after 12 min. The times were regulated by the control unit of the autosampler (Varian 8000). The peak areas were evaluated with a Hewlett-Packard 3290A integrator.

Mass spectrometry

Mass spectra of the derivatives formed were recorded in a Varian MAT 44S gas chromatograph–mass spectrometer with an ionization voltage of 70 eV. The side product of the synthesis of the methylcarbonate oxazolidineone derivative was identified with a Cf-252 plasma desorption mass spectrometer Bio-Ion BIN-10K. The sample was introduced after application on a foil using an electrospray technique.

Reagents and chemicals

Alprenolol, H 56/60 (internal standard for alprenolol), H 74/08 (the oxazolidine-2-one of alprenolol), 4-hydroxy alprenolol (H 104/12) and H 155/48 (the internal standard for the metabolite) were available as hydrochlorides (the amines) from the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). Their structures are shown in Table I. Also from the same source was 4-hydroxy methoxyethyl benzene.

Phosgene (2 *M*) in toluene was from Fluka (Buchs, Switzerland).

The marker trichloroethylcarbamate of dibenzylamine was prepared as previously described[14].

Sodium phosphates were used for the buffers (μ = 1).

Methods

Evaluation of reaction conditions. Typically, the procedure was as follows: buffer (1 ml, μ = 1), water or urine (0.5 ml) and a solution of the compounds of interest (usually 5 μg of alprenolol and 10 μg of the hydroxy compounds) were mixed and derivatized with three 20-μl aliquots of 2 *M* phosgene in toluene. The reagent was added at 2-min intervals. Dichloromethane (2 ml) with marker (13 μg) was added

TABLE II

INFLUENCE OF URINE ON THE DERIVATIZATION OF ALPRENOLOL AND 4-HYDROXY ALPRENOLOL WITH PHOSGENE IN A TWO-PHASE SYSTEM

Using pH 12.3 buffer; water added to keep the volume constant; for rest of conditions, see text.

Sample		*Yield*		
		Alprenolol	*4-Hydroxy alprenolol*	*H 155/48 (internal standard)*
Aqueous*		99	93	96
		101	107	104
Urine	50	101	110	97
(μl)	100	103	84	76
	250	102	17	8
	500	107	–	–
	750	104	–	–
	1000	102	–	–

* The average of the two aqueous samples was arbitrarily set to 100%.

before or after the derivatization step as indicated. The organic phase was isolated and mixed with methanol (1 ml). After 1 h, the solvents were removed in a stream of nitrogen. The residue was taken up in ethyl acetate and subjected to gas chromatography. Any deviations in the procedure are indicated in the Results and discussion section. Peak-area ratios *versus* the marker were calculated and presented, as indicated in Tables II–V.

TABLE III

DERIVATIZATION OF ALPRENOLOL AND 4-HYDROXY ALPRENOLOL WITH PHOSGENE AND METHANOL IN A TWO-PHASE SYSTEM

The reaction was performed throughout with 1 ml of methanol after the phosgene derivatization; for rest of conditions, see text.

Sample		*Yield*		
		Alprenolol	*4-Hydroxy alprenolol*	*H 155/48 (internal standard)*
Aqueous, no methanol		99	102	99
		101	98	101
Aqueous, 50 μl of methanol		105	109	111
		103	107	108
Urine + methanol	50	95	51	37
(μl)	100	91	55	40
	200	88	54	36
	400	76	58	33

TABLE IV

DERIVATIZATION OF ALPRENOLOL AND 4-HYDROXY ALPRENOLOL IN A TWO-PHASE SYSTEM WITH PHOSGENE AND METHANOL USING DIFFERENT MODES OF METHANOL TREATMENT

For conditions, see text.

Sample	*Methanol*		*Yield*	
	50 µl	*1 ml (after the phosgene reaction)*	*Alprenolol**	*4-Hydroxy alprenolol***
Aqueous	–	+	100	29
			100	30
Aqueous	+	–	99	92
			97	88
Aqueous	+	+	102	102
			102	98
Urine	+	–	77	66
			73	72
Urine	+	+	75	78
			71	85

* The average of the six highest yields was arbitrarily set to 100%.
** The average of the two highest yields was arbitrarily set to 100%.

TABLE V

COMPARISON BETWEEN SINGLE-PHASE AND TWO-PHASE DERIVATIZATION OF ALPRENOLOL AND 4-HYDROXY ALPRENOLOL WITH PHOSGENE AND METHANOL

For conditions, see text.

Sample	*Yield*	
	*Alprenolol**	*4-Hydroxy alprenolol***
Aqueous, single-phase	95	88
	93	91
Aqueous, two-phase	101	20
	102	23
Urine, single-phase	98	96
	100	104
Urine, two-phase	86	74
	86	73

* The average of the four highest yields was arbitrarily set to 100%.
** The average of the two highest yields was arbitrarily set to 100%.

Determination of alprenolol and 4-hydroxy alprenolol in urine. The urine sample (0.5 ml) was mixed with 1 ml of buffer (pH 12.3), 50 μl of methanol, 150 μl of an internal standard solution (H 56/60 and H 155/48) and water to make a total volume of 2 ml. Phosgene (2 *M*) was added, with vigorous shaking, via a press-button repeating syringe (Hamilton, Bonaduz). Aliquots (20 μl) were added at 0, 2, and 4 min. After 6 min, 2 ml of dichloromethane containing the marker were added and the shaking was continued for at least 2 min. The separated organic phase was then evaporated. The residue was dissolved in 150 μl of ethyl acetate. A 3-μl aliquot was injected via the autosampler into the gas chromatograph.

RESULTS AND DISCUSSION

Overall strategy

Earlier experience with phosgene derivatization of the isopropylaminopropanol side-chain of β-blockers[4] and their metabolites[5,6] have shown that the reaction can be performed in an aqueous system[4–6]. The optimal pH range was 7.5–12.1 for metoprolol[4] and 11.7–12.5 for the amino acid metabolite H 117/04 of metoprolol[5]. In the final method for metoprolol, however, a two-phase system was preferred to avoid gel formation of the plasma proteins present. Derivatization of the amino acid metabolite required 60 μl of 2 *M* phosgene, as compared with only 10 μl for metoprolol in the two-phase system.

With the presence of a phenol group in the compound to be derivatized, it was assumed that a reactive chloroformate would be formed that was prone to react with water or other compounds present in the sample. Therefore, the first experiments were performed in a two-phase system, where the formed derivative could be extracted into the organic phase and thus be more protected against hydrolysis or side reactions.

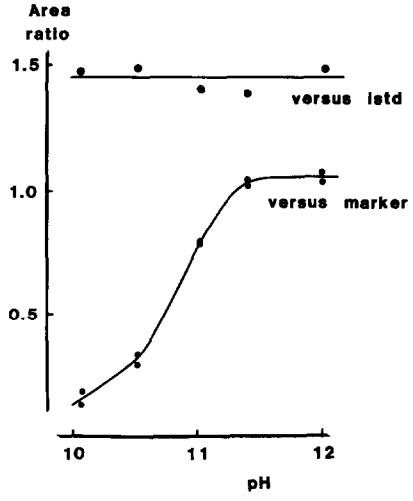


Fig. 1. The pH dependence of the derivatization of 4-hydroxy alprenolol in urine with phosgene and methanol. (For conditions see text.)

Evaluation of reaction conditions for the derivatization of 4-hydroxy alprenolol to an oxazolidineone carbonate

The reaction conditions studied were as follows: influence of pH (Fig. 1), presence of urine in the 2-phase system (Table II), derivatization with methanol present (Table III and IV) and single phase *versus* two-phase derivatization (Table V).

Optimal pH for the derivatization, using 4-hydroxy methoxyethyl-benzene as the model phenol compound, was found to be in the range 11–12 (6–12 studied) in a two-phase system with dichloromethane and phosphate buffer. At the lower pH end, the yield of the chloroformate formed decreased, and the unreacted phenol increased. The corresponding chlorocarbonyl derivative (chloroformate) of 4-hydroxy alprenolol could not be gas-chromatographed with the present system except after treatment with an alcohol such as methanol. The initial pH-dependence of the yield was similar to that of the model above, although the yield tended to decrease at pH $\leqslant$ 11 (Fig. 1). The pH chosen was 12. The pH after the reaction was $<$ 10. At least 1 h with 33% methanol was required for the methyl carbonate formation. Structures of the derivatives are shown in Table I.

When the derivatization procedure with the two-phase system was performed in the presence of increasing volumes of urine, the yield fell when the urine content was $\geqslant$2.5% (Table II). Meanwhile, the yield of the alprenolol derivative was not affected (Table II). The yield in the presence of urine instead of water became acceptable when methanol was included in the system from the start. The percentage methanol present has little influence on the yield of the 4-hydroxy alprenolol derivative but appears to affect alprenolol in a negative way (Table III). A 50-μl aliquot of methanol was chosen as a suitable amount for 2 ml of aqueous reaction solution, including 25% urine. The second methanol treatment is therefore now superfluous (Table IV). It was then investigated whether a single-phase (absence of dichloromethane) or a two-phase derivatization system was to be preferred. The results are given in Table V. These results show that with urine present, the best yield is obtained with the single-phase system.

Selection of internal standards

The reaction conditions were evaluated with the aid of a marker. A presumptive internal standard for 4-hydroxy alprenolol, H 155/48, was also included at an early stage of method development, as the delicate nature of the derivatization reaction would probably require a reacting internal standard having a closely related chemical structure (Table I). An internal standard for alprenolol (H 56/60, Table I) was also available and was included in the final method.

From the upper curve in Fig. 1, the peak area ratio of 4-hydroxy alprenolol to its internal standard is plotted and found to be constant over the pH range studied. Thus, the internal standard will compensate for deviations in the initial pH, although the yield will diminish at lower pH, as observed from the curve of calculations *versus* the marker.

Standard curves and precision of the method

Standard samples were prepared with a urine blank in the range 0.16–10 μg/ml of alprenolol and 0.32–20 μg/ml of metabolite, plus blanks with and without internal standards. The samples were analysed according to the *Determination of alprenolol*

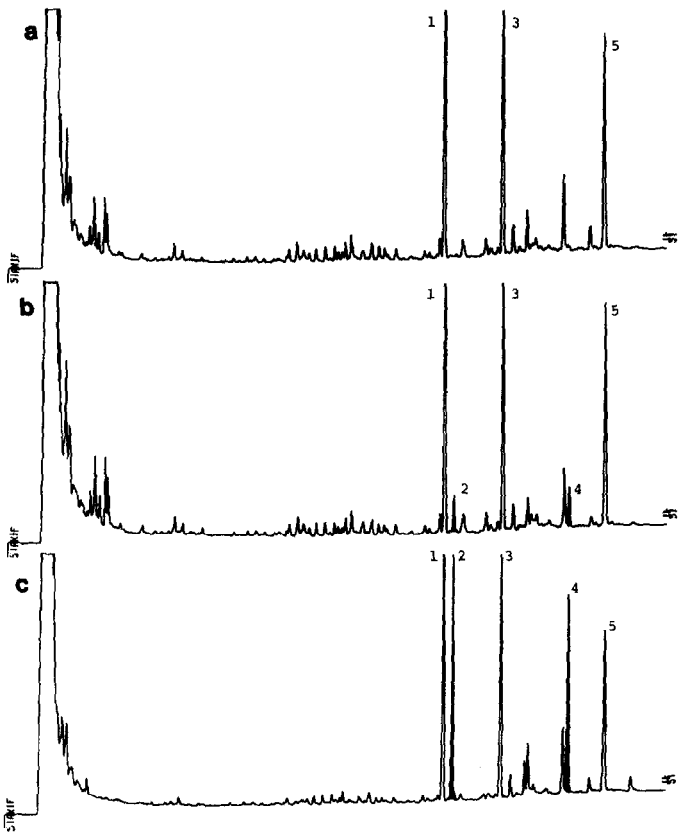


Fig. 2. Gas chromatograms showing the analysis of alprenolol and 4-hydroxy alprenolol in urine after derivatization with phosgene and methanol. (For conditions see text.) (a) urine blank, (b) urine with 0.83 and 1.66 μg/ml and (c) 10 and 20 μg/ml of alprenolol and metabolite added. 1 = internal standard for alprenolol, 2 = alprenolol, 3 = marker, 4 = 4-hydroxy alprenolol and 5 = internal standard for the metabolite (retention time 10.9 min).

and 4-hydroxy alprenolol in urine section, and calibration curves were constructed by plotting peak-area ratios *versus* concentration. These plots were linear in the range studied.

The precision upon repeated analyses of spiked urine samples were 1.4 and 1.7% at the 6.7 and 13.3 μg/ml levels of alprenolol and 4-hydroxy alprenolol. Corresponding values at the 1.7 and 3.3 μg/ml levels were 3.4 and 2.1%, respectively (n = 8). When urine from six individuals were spiked at the higher levels, the precision was virtually the same (1.1 and 1.8) as when the internal standards were used, but *ca.* 21% for 4-hydroxy alprenolol when the marker was used.

Chromatograms showing the analysis of spiked urine samples are given in Fig. 2, including a urine blank with the internal standards and the marker.

Absolute yield of spiked urine samples

The absolute yield of alprenolol in urine at the 10 μg/ml level was 84% when

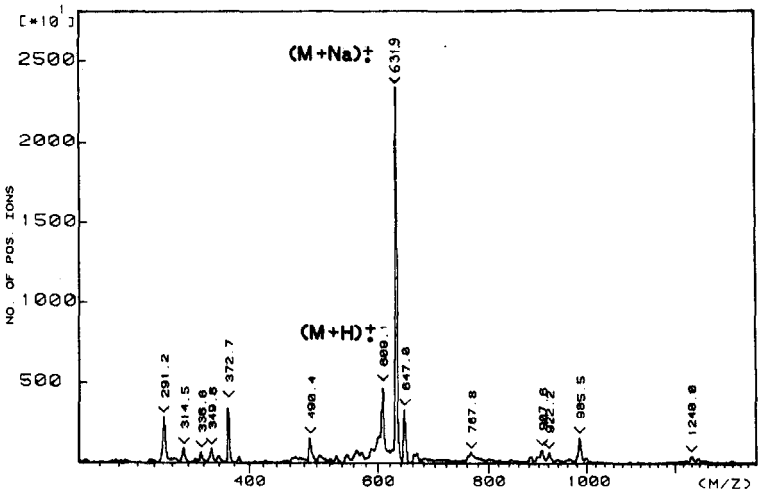


Fig. 3. Cf-252 plasma desorption mass spectrum of bis(4-hydroxy alprenolol) carbonate.

compared with the pure derivative as reference. This value is in line with previous results[4,5].

Attempts to synthesize the oxazolidineone methyl carbonate derivative of 4-hydroxy alprenolol failed due to the formation of bis(4-hydroxy alprenolol) carbonate as a side product. It was not possible to separate the side product from the desired derivative by preparative column chromatography on silica gel, as they were eluted together with 10% methanol in dichloromethane. Therefore, the yield was estimated by using alprenolol oxazolidineone as reference, assuming that they give equal signals on a weight basis in the flame ionization detector. The absolute yield was determined to 24% at the 10 μg/ml level in urine. A comparison with the gas chromatogram of the impure synthesis product and a closer look at the chromatogram, after derivatization of 4-hydroxy alprenolol alone, revealed the presence of a peak with a shorter retention time (*ca.* 0.7 min) than the methyl carbonate derivative of 4-hydroxy alprenolol. Mass spectral analysis after gas chromatographic separation revealed the presence of 4-hydroxy alprenolol oxazolidineone. This fact was contradicted by chemical treatment, as the peak was unaffected by silylation or acylation reagents that normally react with phenols. However, this peak decreased when the injector temperature was lowered. Cf-252 plasma desorption mass spectrometry with an ionization chamber kept at room temperature[15] gave the expected molecular ion with H^+ (609) and an intense molecular ion with sodium added (631) (Fig. 3). Later, direct-inlet conventional mass spectrometry confirmed the molecular weight (608) of the side product. These discouraging results show that the alprenolol oxazolidineone chloroformate is more reactive with 4-hydroxy alprenolol than with methanol, although the latter is present in large excess.

Some supplementary experiments were then performed. Methyl chloroformate (5–50%, 0.6–6 *M*) was added to the 2 *M* phosgene in toluene reagent and, as before, 60 μl of this mixture was used for the derivatization. The yield was *ca.* 50% better with 25 and 50% inclusion of methyl chloroformate. Some methyl carbamate (*ca.*

7% of the methyl carbomate peak area) was observed in the chromatograms, although previous experiments had shown that with equimolar concentrations of phosgene and methyl chloroformate only traces of carbamate were formed from alprenolol. This indicated that phosgene is more reactive than methyl chloroformate. Thus, using 25% methyl chloroformate in the phosgene reagent, the absolute yield of spiked urine could be improved to 45%. Simultaneously, the chromatographic background increased.

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