Journal of Chromatography, 413 (1987) 270–276
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3406

Note

Derivatization of 2-amino alcohols with phosgene in aqueous media: limitations of the reaction selectivity as found in the presence of O-glucuronides of alprenolol in urine

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(First received June 19th, 1986; revised manuscript received September 4th, 1986)

Derivatization of amino alcohols with phosgene to oxazolidinones has found several practial analytical applications [1–5] in both gas and liquid chromatographic analyses. Per se, this cyclic derivative formation should give a certain degree of selectivity as monofunctional compounds will not form cyclic derivatives [6] but are more likely to react with the sample matrix or show poor chromatographic characteristics [1].

The presence of other functional groups, such as hydroxy or carboxyl, presents no problems when derivatizing with phosgene, although they must be silylated before gas chromatography [3]. Phenols, however, are more reactive than alcohols with phosgene in the alkaline aqueous system used. In the presence of methanol and phosgene a methyl carbonate is formed from the phenol group [7]. The symmetric phenol carbonate is also formed to a considerable extent as a side-product [7]. This derivatization procedure was developed for the determination of alprenolol and its phenol metabolite 4-hydroxyalprenolol added to blank urine. The method worked well in the lower μg/ml range [7].

Application of this method to urine from healthy volunteers on an alprenolol regimen showed that both alprenolol and its main metabolite could be found as derivatives, although any free concentrations of these compounds had been eliminated by pre-extraction of the urine samples. This paper discusses and attempts to elucidate the underlying chemistry based on some further experiments, and describes how the problem can be circumvented and the selectivity of the method controlled.

0378-4347/87/$03.50

EXPERIMENTAL

Gas chromatography

The instrumental set-up was virtually the same as in previous work [7]. Some details of immediate interest are given in Fig. 3.

Reagents and chemicals

Alprenolol, H 56/60 (internal standard for alprenolol, I), 4-hydroxyalprenolol (H 104/12) and H 155/48 (internal standard for the metabolite, II) as hydrochlorides and the oxazolidine-2-one of metoprolol used as a marker (H 151/38 [1]) were obtained from the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). The structures were given earlier [7]; those of alprenolol and the metabolite are shown in Fig. 1.

Phosgene (2 *M*) in toluene was purchased from Fluka (Buchs, Switzerland). Methyl chloroformate (zur Synthese) was obtained from Merck (Darmstadt, F.R.G.) and had been glass-distilled. Bis(trimethylsilyl)acetamide (BSA) was obtained from Macherey & Nagel (Düren, F.R.G.). Sodium acetate (pH 4.5), carbonates (pH 10) and phosphates (pH 12) were used as buffers (ionic strength 1). β-Glucuronidase solution was obtained from Sigma (St. Louis, MO, U.S.A.).

Urine samles were made available from the Department of Clinical Pharmacology (Cardiovascular), AB Hässle. They were collected from male volunteers who had been given alprenolol benzoate (Aptin CR) 260-mg controlled release tablets.

Methods

Determination of alprenolol and 4-hydroxyalprenolol in urine. The urine sample (0.5 ml) was mixed with 1 ml of buffer (pH 12.3), 50 μl of methanol and 150 μl of a solution containing the internal standards I and II (33 and 67 μg/ml, respectively). Phosgene was added with vigorous shaking with the aid of a press-button repeating syringe (Hamilton, Bonaduz, Switzerland). Aliquots (20 μl) were added at 2-min intervals; total volume added was 60 μl. After 6 min, 2 ml of dichloromethane containing 2 μg/ml of the marker were added and agitation was continued for 2 min. After centrifugation, the aqueous phase was aspirated off and the remaining organic phase decanted into an injection vial and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.5 ml of ethyl acetate. A 3-μl aliquot was injected with an autosampler into the gas chromatograph with a capillary column and flame ionization detector.

Calibration graphs were constructed after analysing blank urine with multiple volumes (1–10) of 50 μl (Eppendorf repeater pipette, Hamburg, F.R.G.) containing both added alprenolol and the 4-hydroxy metabolite. The peak-area ratios were plotted versus the respective standard concentrations.

Determination of free concentrations in actual urine samples. The urine sample was mixed with an equal volume of buffer (pH 10) and extracted with a five times larger volume of ethyl acetate. An aliquot of the organic phase was evaporated to dryness and then analysed as above after adding 0.5 ml of blank urine.

Determination of total concentrations in actual urine samples. The total con-

centration of alprenolol and 4-hydroxyalprenolol was determined after treatment with β-glucuronidase. A 0.2-ml sample was mixed with 0.3 ml of buffer (pH 4.5) and 0.15 ml of the internal standard mixture. After addition of 50 μl of enzyme solution the mixture was incubated for 24 h at 37°C in a thermostated water-bath. Then 1 ml of buffer (pH 12.3) and 5 *M* sodium hydroxide was added to give a final pH of 12.0 ± 0.1 and the sample was ready for derivatization with phosgene–methanol as above.

The bound fraction was determined after pre-extraction of the free fraction as described above.

Derivatization of alprenolol conjugate with methyl chloroformate, enzymatic hydrolysis and analysis for liberated methyl carbamate of alprenolol. A urine sample (0.5 ml) with no free alprenolol present was made alkaline with 1 ml of 0.5 *M* sodium carbonate solution, then 50 μl of 100 μl/ml I (internal standard solution) were added. Next, the sample was derivatized with 3×10 μl of methyl chloroformate followed by extraction with dichloromethane to remove any liberated alprenolol carbamate. The aqueous phase was mixed with 1 ml of acetate buffer and 200 μl of 5 *M* acetic acid to give a final pH of 4.5 ± 0.2. After addition of 100 μl of enzyme solution the mixture was incubated as above. The extraction was performed with 5 ml of dichloromethane containing 4 μg of the marker. The organic phase was collected, evaporated carefully and silylated with BSA (20 μl) for at least 5 min before addition of 0.5 ml of ethyl acetate and gas chromatography. A reference sample with 5 μg of alprenolol and the internal standard in blank urine was run simultaneously.

RESULTS AND DISCUSSION

Metabolism of alprenolol

The biotransformation of alprenolol has been extensively studied [8,9]. In man the 4-hydroxylated metabolite accounted for ca. 80% of the dose excreted, the major part being eliminated in the form of glucuronides. The presence of both alcoholic and phenolic glucuronides was shown by treatment with chloromethyldimethylchlorosilane [10] followed by enzymatic hydrolysis and then trimethylsilylation before gas chromatography [9].

Derivatization with phosgene and methanol and lack of selectivity of the reaction

Application of the phosgene–methanol derivatization reaction [7] to actual urine samples with any free alprenolol and the 4-hydroxy metabolite removed by extraction gave unexpectedly significant amounts of these two compounds, although not as much as could be found after enzymatic hydrolysis of the sample prior to the derivatization reaction.

The derivatization of alprenolol and its phenolic metabolite is assumed to proceed via chlorocarbonylation of the amine and the phenol groups. The chlorocarbonylamine then cyclizes with the hydroxy group, giving an oxazolidine-2-one, while the aromatic chloroformate from the phenol reacts with methanol present to give a methyl carbonate or to a significant extent, although not desired, with other phenols present in the system including 4-hydroxyalprenolol. In the last

instance a symmetrical carbonate is formed, which decomposes to a phenol in the injector of the gas chromatograph [7]. The normal reaction is outlined in Fig. 1a and b. The side-reaction is indicated in the lower part of Fig. 1b. The alcoholic glucuronide of alprenolol (and 4-hydroxyalprenolol) also forms the oxazolidine-2-one when reacted with phosgene. It is suggested that the initially formed chlorocarbonylamine derivative attacks the glucuronide ether oxygen and the oxazolidine-2-one is formed. Meanwhile, the chlorocarbonyl moiety may also attack other species in the urine sample, which explains why the cyclization is not complete. The proposed scheme is shown in Fig. 1c.

Urine samples were analysed for alprenolol and 4-hydroxyalprenolol both directly without any prior treatment and after enzymatic hydrolysis with β-glucuronidase to give the total concentration of the two compounds present. The relationship is illustrated in Fig. 2. The slope is less than unity, which would have been the case if the conjugates reacted to the same extent as the free compounds. Also, the slope of the relationship 4-hydroxyalprenolol "total" versus "direct" is less than that for alprenolol, probably because the phenol can also be conjugated [9] and this ether linkage is not as easily attacked by phosgene.

Derivatization of alprenolol conjugate with methyl chloroformate, enzymatic hydrolysis and trimethylsilylation before gas chromatography

A further support for the hypothesis that phosgene reacts with the secondary amine of hydroxylic alprenolol glucuronide was found by reacting the conjugate with methyl chloroformate. The assumed alprenolol carbamate glucuronide was then treated with β-glucuronidase and the liberated alprenolol carbamate was isolated and trimethylsilylated before analysis by gas chromatography. As illustrated in Fig. 3b, the peak corresponding to alprenolol carbamate (peak 2) is liberated by the enzymatic treatment as no significant peak is visible in Fig. 3a at this retention time, that is, the extract just after the methyl chloroformate reaction and before enzymatic hydrolysis. This reaction route (Fig. 4) using chloroformate derivatization and enzymatic hydrolysis is a new and alternative way of characterizing alcoholic glucuronides of β-blocking drugs. It would also be possible to use this approach to determine the proportion of phenolic and hydroxylic conjugation of 4-hydroxyalprenolol.

Restoration of the method selectivity

Owing to the lack of selectivity in the phosgene derivatization reaction, the free concentrations cannot be determined directly by this method [7]. Instead, isolation of the free fractions by extraction at pH 10 [9] before derivatization is recommended. The bound fraction can then be liberated by enzymatic hydrolysis.

Free alprenolol was only found in one of the samples analysed (0.6 μg/ml), whereas the metabolite was present in all (4–18 μg/ml), except for one person with no (less than 0.1 μg/ml) free or bound metabolite. Such a hydroxylation deficiency is found in about 10% of the Swedish population [11] but so far no detailed study has appeared.

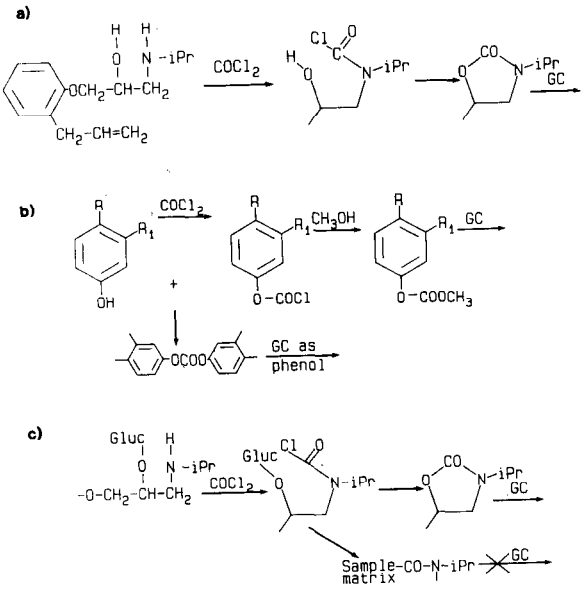

Fig. 1. (a) Scheme of the reaction of alprenolol with phosgene to an oxazolidineone. (b) Scheme of the reaction of the phenol group of 4-hydroxyalprenolol with phosgene and methanol including the side-reaction of the intermediate chloroformate with the original phenol (lower part). (c) Proposed reaction scheme for the formation of oxazolidineone from alprenolol O-glucuronide and phosgene.

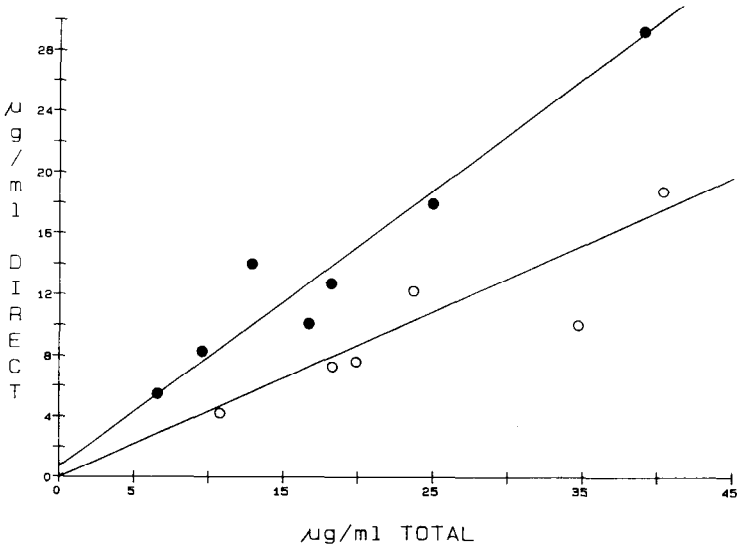

Fig. 2. Comparison between "direct" determination of alprenolol and 4-hydroxyalprenolol and determination after enzymatic hydrolysis of urine samples ("total"). (●) Alprenolol; (○) 4-hydroxyalprenolol. Linear regression analysis: for alprenolol, correlation coefficient 0.966, slope 0.69, intercept 1.4 µg/ml; for 4-hydroxyalprenolol, 0.879, 0.40 and 0.07, respectively.

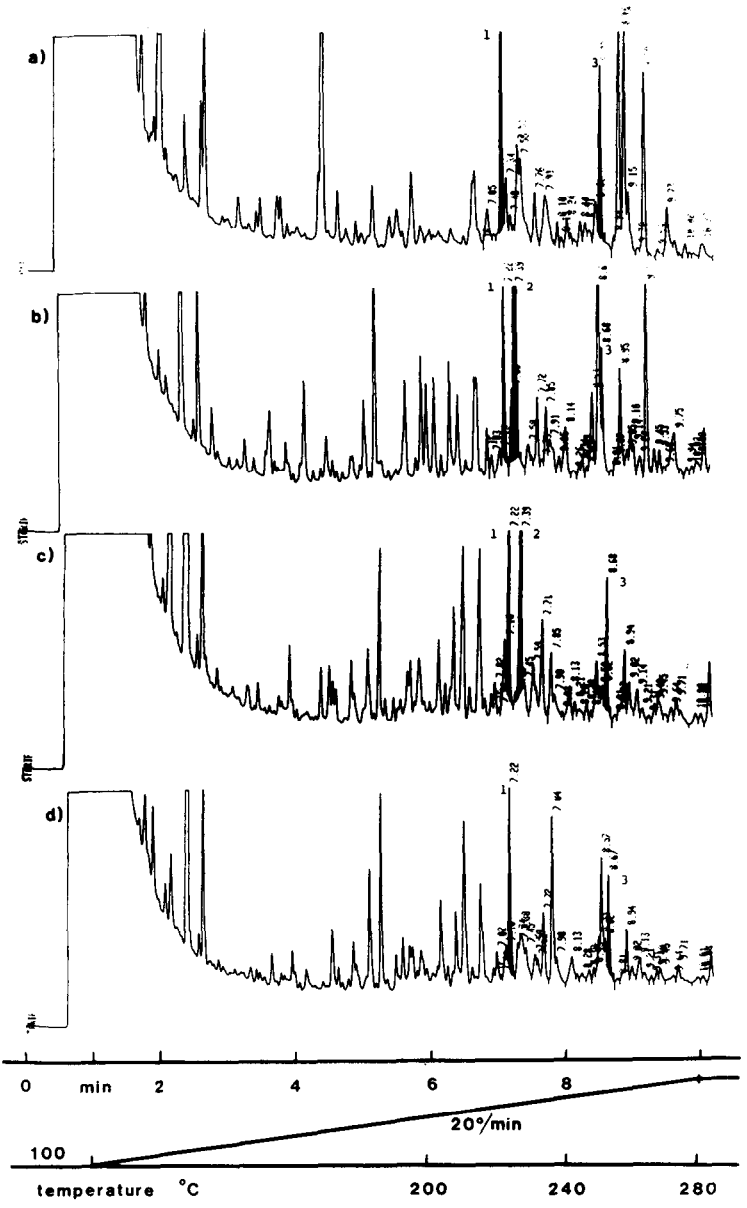

Fig. 3. Gas chromatograms for the analysis of a urine sample for alprenolol. (a) Methyl chloroformate derivatization; (b) methyl chloroformate derivatization followed by enzymatic hydrolysis; (c) blank urine with 5 μg of alprenolol added and treated as b; and (d) blank urine with internal standard and marker. All samples trimethylsilylated (BSA) before GC. GC conditions: 25 m $\times$ 0.32 mm I.D. fused-silica column with 5% phenylmethylsilicone; 100 kPa nitrogen; oven temperature, 1 min at 100°C, then increased at 20°C/min to 280°C; splitless, 1 min. Peaks: 1 = internal standard I (7.22 min); 2 = alprenolol (7.39 min); 3 = marker (8.68 min).

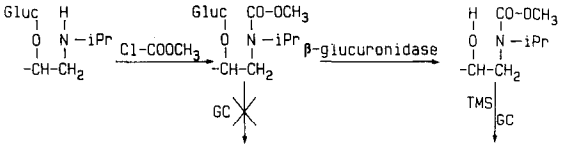

Fig. 4. Proposed reaction scheme for the formation of a methylcarbamate of alprenolol from alprenolol O-glucuronide.

ACKNOWLEDGEMENTS

The author expresses his sincere gratitude to Professor Jörgen Vessman and Dr. Kurt-Jürgen Hoffman for valuable discussions and encouragement.

REFERENCES

1 O. Gyllenhaal and J. Vessman, J. Chromatogr., 273 (1983) 129.
2 I. Wainer, T.D. Doyle, K.H. Donn and J.R. Powell, J. Chromatogr., 306 (1984) 405.
3 O. Gyllenhaal and K.-J. Hoffmann, J. Chromatogr., 309 (1984) 317.
4 W.A. König, K. Ernst and J. Vessman, J. Chromatogr., 294 (1984) 423.
5 J. Hermansson, J. Chromatogr., 325 (1985) 379.
6 C.F. Poole and A. Zlatkis, J. Chromatogr., 184 (1980) 99.
7 O. Gyllenhaal, J. Chromatogr., 349 (1985) 447.
8 N.-O. Bodin, Life Sci., 14 (1974) 685.
9 K.-J. Hoffmann, A. Arfwidsson, K.-O. Borg and I. Skånberg, Biomed. Mass Spectrom., 5 (1978) 634.
10 C.G. Hammar, Biomed. Mass Spectrom., 5 (1978) 25.
11 C.-G. Regårdh, personal communication.