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Chiral chloroformates as transparent reagents for the resolution of metoprolol enantiomers by reversed-phase liquid chromatography

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SUMMARY

Ten chiral chloroformates were prepared and tested as derivatizing reagents, using metoprolol and tyrosine enantiomers as model compounds. Chloroformates derived from isosorbide and isomannide monoesters gave separation factors of 1.07–2.16 for tyrosine and 1.06–1.08 for metoprolol in reversed-phase liquid chromatography. With *tert.*-butyl 3-(chloroformoxy)butyrate as derivatizing reagent a separation factor of 1.10 and a resolution of 2.0 were obtained in 12 min for (*R*)- and (*S*)-metoprolol. The reagent and its by-products did not interfere with the UV or fluorescence detection of metoprolol.

INTRODUCTION

Chloroformates have chemical properties that make them attractive as derivatizing reagents for primary and secondary amines. They have also found use in the derivatization of tertiary amines and alcohols and as coupling agents for the condensation of carboxylic acids with amines. Their reactivity with primary and secondary amines allows derivatization under mild conditions in the presence of water. Chloroformates are not harmful to common reversed-phase chromatographic columns.

So far, only two chiral chloroformate reagents have been reported as derivatizing reagents for the analytical resolution of enantiomers[1]. Menthyl chloroformate has been used for gas chromatographic separations[2] and for the liquid chromatographic resolution of hydroxy acids[3] and some secondary amines[4,5]. 1-(9-Fluorenyl)-ethyl chloroformate (FLEC) was introduced as a chiral derivatizing reagent by Einarsson *et al.*[6], who demonstrated the simultaneous resolution of seventeen amino acids into their D- and L-forms as diastereomeric derivatives. The fluorenyl residue allowed sensitive fluorimetric detection.

The work reported here was carried out in a search for chiral chloroformate reagents suitable for the resolution of metoprolol and other β-blockers with the N-isopropylamino-2-propanol structure. These have their chiral carbon atom in

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a β-position to the amino group. Ten different chloroformates (Table I) were prepared and tested, using (*S*)- and (*R*, *S*)-metoprolol and, for seven of the reagents, L- and DL-tyrosine as model compounds. As β-blockers can be sensitively detected by fluorescence, it was considered an advantage if the reagent itself does not contain fluorescent or UV-absorbing groups.

EXPERIMENTAL

Chemicals

Racemic 1-(cyclohexyl)ethanol, 3-hydroxytetrahydrofuran and (−)-menthyl chloroformate were obtained from Aldrich (Milwaukee, WI, U.S.A.), (*R*)-methyl 3-hydroxybutyrate and (*S*)-ethyl 3-hydroxybutyrate from Fluka (Buchs, Switzerland) and (*S*)-*tert.*-butyl 3-hydroxybutyrate from Merck (Darmstadt, F.R.G.). Isosorbide 2-mononitrate, 5-mononitrate and 2-monoacetate, isomannide mononitrate, (*R*, *S*)- and (*S*)-metoprolol, (*R*, *S*)-N-desisopropyl metoprolol and (*R*, *S*)-N-(2-hydroxypropyl) acetamide were obtained from the Organic Chemistry Department, AB Hässle (Mölndal, Sweden). L-Tyrosine and DL-tyrosine were from Sigma (St. Louis, MO, U.S.A.).

Chromatographic equipment

The liquid chromatograph consisted of a Model 7010 manual loop injector (Rheodyne, Berkeley, CA, U.S.A.), a pump for isocratic elution, a Model 440 UV detector (Waters Assoc., Milford, MA, U.S.A.), a Model LS4 spectrofluorimetric detector (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The column was 100 mm × 4.6 mm I.D., packed with 3-μm Microspher octadecylsilica particles (Chrompack, Middelburg, The Netherlands).

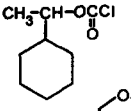
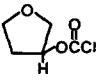
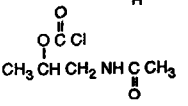
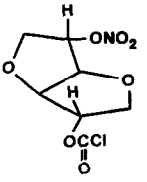
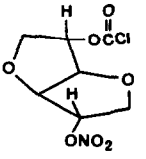
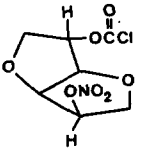
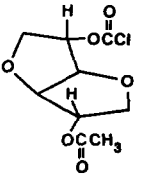
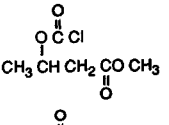
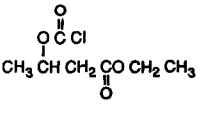
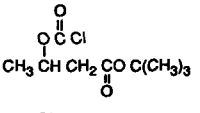
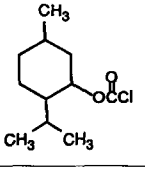
Preparation of chloroformate reagents

Chloroformates 1–10 were prepared as follows: the corresponding alcohol (0.5–5 mmol) and an equimolar amount of triethylamine were dissolved in toluene or dry diethyl ether and the solution was added to cold (0°C) toluene containing 20% (w/w) of phosgene (four-fold excess). The mixture was stirred at 0°C for 2 h, then the solid triethylammonium chloride was removed by filtration and the solvent was evaporated in a rotary evaporator.

The optical purity of the non-racemic reagents 4–7 and 10 was investigated by derivatization with (*S*)-metoprolol (optical purity >99.8%) and racemic metoprolol, followed by liquid chromatographic separation of the diastereomers formed. With reagents 4–7, no enantiomeric impurity was detected (optical purity >99%). Reagent 10 was 94% optically pure (see Fig. 1B), which probably reflects the optical purity of the starting material. Reagent 10 was also analysed by liquid chromatography with UV detection at 220 nm and was found to contain 10% of the parent alcohol. No characterization was carried out that required further purification of the chloroformate reagents. No decomposition of the reagents (neat or dissolved in dichloromethane) was observed after 1 week at +8°C or 1 month at −18°C.

TABLE I

STRUCTURAL FORMULAE OF CHIRAL CHLOROFORMATE REAGENTS

No.	*Compound*	*Formula*
1	(*R*, *S*)-1-(Cyclohexyl)ethyl chloroformate	
2	(*R*, *S*)-Tetrahydro-3-furanyl chloroformate	
3	(*R*, *S*)-N-[2-(Chloroformoxy)propyl]acetamide	
4	2-Chlorocarbonyl-L-isosorbide 5-mononitrate	
5	5-Chlorocarbonyl-L-isosorbide 2-mononitrate	
6	5-Chlorocarbonyl-L-isomannide 2-mononitrate	
7	5-Chlorocarbonyl-L-isosorbide 2-monoacetate	
8	(*R*)-Methyl 3-(chloroformoxy)butyrate	
9	(*S*)-Ethyl 3-(chloroformoxy)butyrate	
10	(*S*)-*tert.*-Butyl 3-(chloroformoxy)butyrate	
11	(−)-Menthyl chloroformate	

Derivatization of amines

To 20 μmol of reagents 1–10, dissolved in 0.20 ml of acetonitrile, were added 0.4 ml of 0.25 *M* borate buffer (pH 8) and 0.4 ml of a solution containing up to 0.4 μmol of the amine in 0.01 *M* hydrochloric acid. After 1–60 min at room temperature, the reaction was stopped by addition of 25 μl of 1 *M* hydrochloric acid, and the sample was then ready for injection into the chromatographic column.

Derivatization with (−)-menthyl chloroformate was performed in dichloromethane owing to the low solubility of this reagent in aqueous solutions. Solid metoprolol base (20 μmol) was added to a mixture of the reagent (200 μmol) and triethylamine (200 μmol). After reaction for 30 min at room temperature, aliquots diluted 1000-fold in the chromatographic eluent were injected.

RESULTS AND DISCUSSION

Table II lists the separation factors obtained for derivatives of metoprolol and tyrosine in reversed-phase systems. Different concentrations of the organic solvents were used, and also different solvents, as the same solvent was not optimum for all derivatives. 1-(Cyclohexyl)ethyl chloroformate resolved neither metoprolol nor tyrosine. Tetrahydro-3-furanyl chloroformate did not resolve metoprolol, but did resolve tyrosine ($\alpha = 1.16$). N-[2-(Chloroformoxy)propyl] acetamide resolved tyrosine ($\alpha = 1.44$), but not metoprolol.

Isosorbides and isomannides are 1,4–3,6-dianhydroglucitols with four chiral atoms and a rigid, non-planar, two-ring structure. Derived from sorbitol and mannitol, they are of high enantiomeric purity. No racemization was observed when pure (*S*)-metoprolol was derivatized. Separation factors for tyrosine ranged from 1.07

TABLE II

SEPARATION OF DIASTEREOMERIC CARBAMATES OF TYROSINE AND METOPROLOL BY REVERSED-PHASE CHROMATOGRAPHY

Column, Microspher C_{18} (3 μm) (100 mm × 4.6 mm I.D.); mobile phase, phosphate buffer (pH 3) ($I = 0.02$) plus an organic solvent.

Reagent[a]	*Tyrosine*				*Metoprolol*			
	Mobile phase[b]	k'_1	k'_2	α	*Mobile phase*[b]	k'_1	k'_2	α
1	20% ACN	9.07		⩽1.01	37% ACN	10.69		⩽1.01
2	10% ACN	9.92	11.49	1.16	37% ACN	5.30		⩽1.01
3	10% ACN	6.5	8.9	1.44	37% ACN	5.1		⩽1.01
4	10% THF	12.32	21.14	1.70	30% THF	19.6	20.8	1.06
5	10% THF	9.51	10.02	1.07	30% THF	23.4	24.4	1.04
6	10% THF	8.12	17.54	2.16	30% THF	15.72	16.93	1.08
7	5% THF	6.74	10.08	1.50	20% THF	21.6	22.9	1.06
8					37% ACN	12.50	13.48	1.08
9					37% ACN	21.41	23.22	1.08
10					50% ACN	10.60	11.63	1.10
11					72% Methanol	5.68	6.38	1.12

[a] See Table I.

[b] ACN = Acetonitrile; THF = tetrahydrofuran.

to 2.16, showing a strong dependence on the orientation (*endo* or *exo*) of the chloroformyl group and the ester group. Separation factors for metoprolol were modest and fairly uniform. Separation factors of 1.04–1.08 were obtained when tetrahydrofuran was used as an organic modifier. Less efficient separations were achieved with mobile phases containing methanol or acetonitrile. Whereas good resolution was obtained for metoprolol ($R = 1.4$), only partial resolution was obtained for the primary amine N-desisopropylmetoprolol ($\alpha = 1.01$–1.03), the best separation being obtained with reagent 4.

The 3-(chloroformoxy)butyrates were chosen for testing as they have a simple chiral structure and contain an ester group that might be available for intramolecular hydrogen bonding of the hydroxyl of metoprolol. They gave equal or higher separation factors for metoprolol than the isosorbide and isomannide reagents. In this instance, acetonitrile gave better resolutions than tetrahydrofuran, methanol or 2-propanol. Using a 100-mm column with 7700 theoretical plates, a resolution factor (R) of 2.0 was obtained (see Fig. 1A).

The menthyl carbamate derivatives of metoprolol were resolved in a chromatographic system similar to that used by Schmitthenner *et al.*[7] for menthyl carbamates of propranolol and flavodilol. The α-value was slightly higher than for *tert.*-butyl 3-(chloroformoxy)butyrate (reagent 10). For applications to biological extracts, the higher water solubility of reagent 10 was considered important. It allowed derivatization in an aqueous solution and injection of the reaction mixture directly into the column.

Detection properties of reagent and derivatives

The molar absorptivity of reagent 4 (1 mM in acetonitrile) at 280 nm was too low to be determined, and was calculated to be lower than 200 l mol^{-1} cm^{-1} at 254 nm. In order to detect reagents 1–11 and the corresponding alcohols in the liquid chromatographic analyses, UV detection below 240 nm had to be used. Under the conditions used for separation of metoprolol derivatives, reagents 1–10 eluted close to the front. Owing to the small disturbance from reagent blanks, sensitive detection of the metoprolol derivatives was easily obtained.

The molar absorptivity (280 nm) of metoprolol derivatives with reagent 4 appeared to be very close to that of metoprolol itself. The measured peak area of the derivatives formed was *ca.* 95% of the decrease in peak area of metoprolol. The peak area of the more retained diastereomer was slightly higher than that for the less retained (a factor 1.03), but this might be due slightly tailing peaks. The fluorescence properties were studied by coupling a UV and a fluorescence detector in series and measuring the ratio of the fluorescence signal over the UV signal for each eluted peak. The fluorescence yields of (S)- and (R)-metoprolol derivatives (reagent 4) were 90% and 95%, respectively, relative to underivatized metoprolol. Similar results were obtained when the UV and fluorescence properties of metoprolol derivatives with reagents 7 and 10 were studied.

Reaction rates and recoveries

When reagent 10 was allowed to react with metoprolol (0.4 μmol in 1.0 ml) under the conditions given under Experimental, liquid chromatography showed a 50% decrease in metoprolol concentration after 4.2 min. With reagent 4, the reaction was

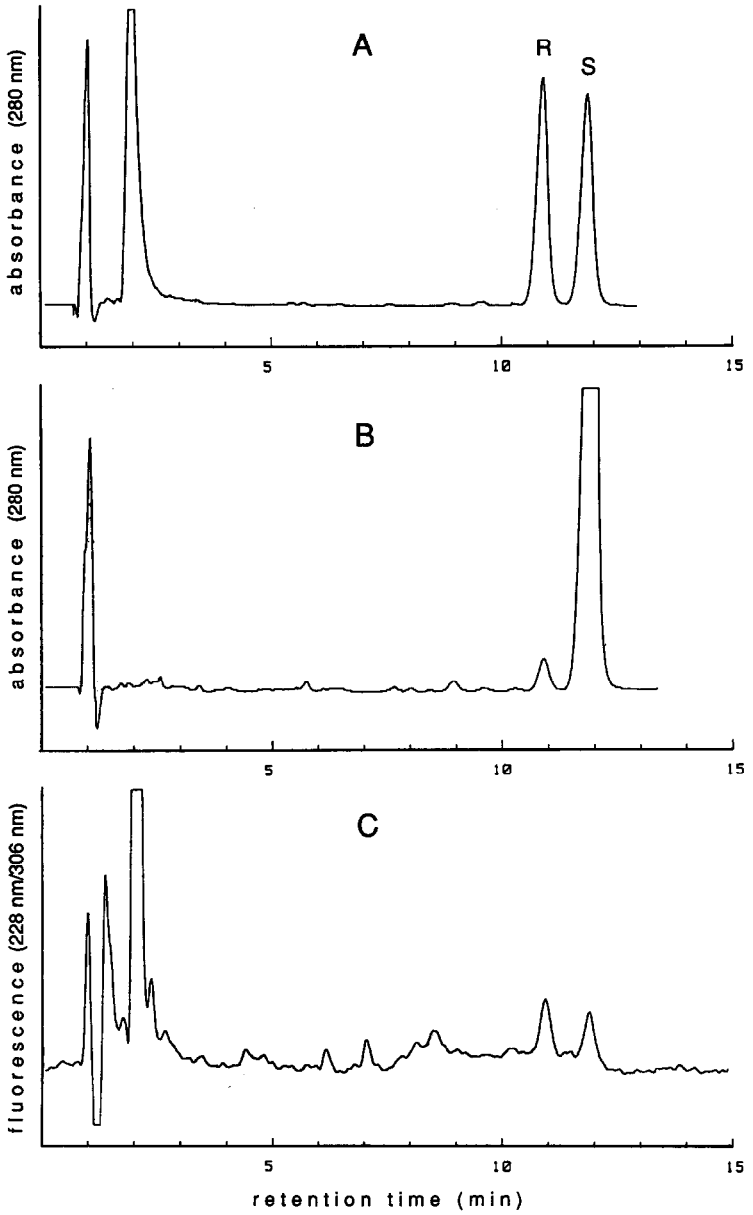


Fig. 1. Separation of (*R*)- and (*S*)-metoprolol after derivatization with *tert.*-butyl 3-(chloroformoxy)-butyrate (reagent 10). (A) Metoprolol racemate. (B) 99.9% Pure (*S*)-metoprolol. The small peak is due to *ca.* 3% enantiomeric impurity of the reagent. (C) Detection of metoprolol enantiomers in human plasma after extraction and derivatization with *tert.*-butyl 3-(chloroformoxy)butyrate. Concentration in plasma *ca.* 4 ng/ml (15 nmol/l) of each metoprolol enantiomer. Chromatographic conditions for A, B and C as given in Table II for reagent 10. In A and B, UV detection at 280 nm was used (0.16 a.u.f.s.); in C, fluorescence was measured at 306 nm (excitation at 228 nm).

about twice as fast. Tyrosine reacted considerably faster than metoprolol with both reagents. When the reactions between metoprolol and reagents 4 and 10 were followed by measuring the diastereomers formed, no difference in the rates of formation could be detected between the diastereomers.

The recovery of metoprolol reacted with reagent 10 (20 m*M*) was found to be independent of the initial metoprolol concentration (0.04–400 μM). It was calculated to be 90–95% with no difference between the enantiomers. An accurate determination of the recovery would require the synthesis of each diastereomer with a purity close to 100%.

Determination of metoprolol enantiomers in plasma extracts

In a preliminary investigation of the potential of the reagents for bioanalytical applications, human plasma (1 ml) containing metoprolol was extracted with a 1:1 mixture of dichloromethane and diethyl ether, according to the method of Balmér *et al.*[8]. The solvent was evaporated and the residue was treated with *tert.*-butyl 3-(chloroformoxy)butyrate (reagent 10) as described above. After a 30-min reaction period, 150 μl of the reaction mixture (1.0 ml) were separated by reversed-phase chromatography under the conditions given in Table I. Fluorescence detection (excitation at 228 or 272 nm, emission at 306 nm) showed little interference from a plasma blank, and 15 nmol/l of each enantiomer of metoprolol could be determined (Fig. 1C). Using the procedure described, α-hydroxymetoprolol, a metabolite, will also be extracted, derivatized and will be eluted in front of the metoprolol derivatives.

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