Journal of Chromatography, 592 (1992) 323–329
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2501

By-products in the derivatization of amines with the chiral reagent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and their elimination

Martin Ahnoff*, Karin Balmér and Yvette Lindman

Bioanalytical Chemistry, Astra Hässle AB, S-431 83 Mölndal (Sweden)

ABSTRACT

The formation of the desired diastereomeric thiourea derivatives did not proceed quantitatively when β-adrenoceptor blocking drugs were allowed to react with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). A competing reaction caused losses, which increased with increasing excess of the reagent (decreasing relative amounts of amine), amounting to as much as 50%. Measurements indicate that the by-products were due to an impurity in the commercial reagent, but also in GITC produced in-house from the corresponding bromide. The side-reaction could be completely eliminated by pretreatment of the reagent solution with a limited amount of another amine. Similar observations of a reactive impurity were made with the chiral reagent α-methylbenzyl isothiocyanate.

INTRODUCTION

Isothiocyanate reagents have been used in peptide sequencing [1] and for amino acid analysis. A number of chiral isothiocyanate reagents have been used as derivatization reagents for the liquid chromatographic separation of amine enantiomers. Neomenthyl isothiocyanate was introduced by Nambara *et al.* [2] and 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC) were introduced by Nimura and coworkers [3,4]. GITC has been used for the separation of enantiomers of β-adrenoceptor blocking drugs and related substances [5,6]. α-Methylbenzyl isothiocyanate [7], 1-(1-naphthyl) and 1-(2-naphthylethyl) isothiocyanate [8] and N-fluorenylmethoxycarbonyl-L-*p*-isothiocyanophenylalanine [9] have also been used as chiral derivatization reagents. The typical use of these reagents, including GITC, has been in the assessment of the enantiomeric composition of chiral amines, but applications to the quantitative determination of enantiomers over a wide concentration range have also been reported [5,8,10–12]. Initial attempts in our laboratory to apply derivatization with GITC to β-blockers in biological samples showed unexpected results and promted us to carry out the present investigation.

EXPERIMENTAL

Chemicals

Metoprolol racemate, (*R*)-(+)-metoprolol, alprenolol, atenolol, propranolol and H 170/37 (N-isopropylamino-2-propanol) were obtained from Organic Chemistry, Astra Hässle (Mölndal, Sweden). GITC was obtained from Polyscience (Warrington, PA, USA) or was synthesized from 2,3,4,6-tetraacetylglucopyranosyl bromide (acetobromoglucose), obtained from Fluka (Buchs, Switzerland). L-α-Methylbenzyl isothiocyanate (MBITC) was from K&K (Costa Mesa, CA, USA) and (*R*)-α-methylbenzyl isocyanate (MBIC) from Aldrich (Steinheim, Germany). 2,3,4,6-Tetraacetylglucopyranosyl isocyanate (GIC) was synthesized analogously to GITC [3].

Liquid chromatography

The liquid chromatograph consisted of a Beck-

man 110A pump, a Rheodyne Model 7125 loop injector with a 5-μl loop, a 100 mm × 4.6 mm I.D. column packed with 3-μm octadecylsilica particles (Nucleosil 120-3 C_{18}, Macherey–Nagel, Düren, Germany), a Spectra-Physics SpectraChrom 100 UV detector and a Perkin-Elmer LS-4 spectrofluorimetric detector coupled in series and two Spectra-Physics Model 4270 integrators. Mixtures of acetonitrile and ammonium acetate buffer (50 mmol/l, pH 6.0) were used as mobile phases (see Table I). UV absorbance was measured at 228 nm. The same wavelength was used for fluorescence excitation, while emission was measured at 306 nm (342 nm for propranolol).

Liquid chromatography–mass spectrometry

A Finnigan MAT (San José, CA, USA) TSQ-70 mass spectrometer with a TSP-1 thermospray interface was used. The vaporizer and ion source temperature settings were 100 and 250°C, respectively. The repeller potential was + 50 V for positive ions and − 50 V for negative ions. Samples (100 μl) were separated on the same column and with the same mobile phase mixtures that were used for UV and fluorescence detection.

Procedures

Solutions of β-blockers (0.50 mmol/l in acetonitrile) were prepared either by direct dissolution of the free base in acetonitrile or by extraction of an aqueous alkaline solution of the salt with diethyl ether–dichloromethane (4:1, v/v), solvent evaporation and reconstitution in acetonitrile. Derivatization was carried out at room temperature by mixing the β-blocker, reagent and triethylamine, all dissolved in acetonitrile. The reaction time was 20 min for GITC, GIC and MBIC and 3 h for MBITC.

RESULTS AND DISCUSSION

Structural formulae of GITC, metoprolol and propranolol and its thiourea derivative are shown in Fig. 1.

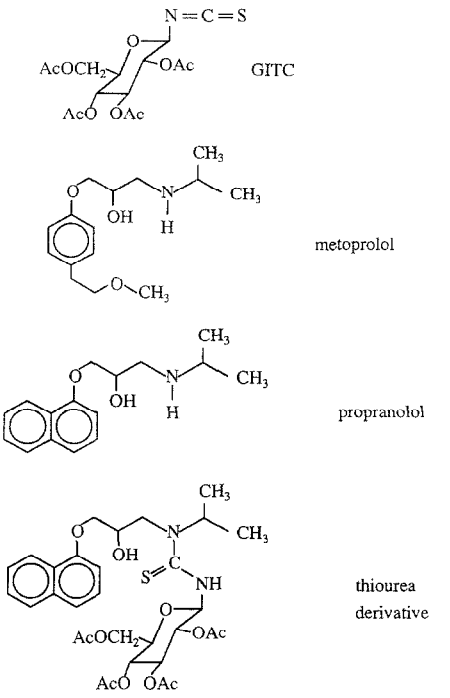

Fig. 1. Structural formulae. Ac = Acetyl.

Chromatography of reaction products: UV and fluorescence detection

When metoprolol racemate (0.02–10 mmol/l) was allowed to react with GITC (10 mmol/l) in the presence of triethylamine (TEA, 11 mmol/l), the reaction was complete within 10 min at room temperature. Fluorescence detection revealed two pairs of peaks. Derivatization of pure (*R*)-(+)-metoprolol showed that the last-eluted peak in each pair was a product of this enantiomer. Two pairs of peaks were also obtained from racemates of alprenolol, atenolol and propranolol. Table I lists the retention data for these products.

Fig. 2 shows UV and fluorescence recordings of the separation of the products of propranolol. In comparison with the UV signal, the fluorescence signal was one order of magnitude higher for products X than products D from propranolol. For metoprolol the difference in the fluorescence to UV signal ratio was even greater, and for reactions with low concentrations of β-blocker it was difficult to determine both types of products with the same detector.

Formation of products X and D under various reaction conditions.

When equimolar concentrations (10 mmol/l) of metoprolol and GITC were allowed to react in the presence of TEA, the portion of derivatives X_1 and

TABLE I

SEPARATION OF REACTION PRODUCTS OF FOUR β-BLOCKERS DERIVATIZED WITH GITC

Stationary phase: Nucleosil 120-3 C_{18}. Mobile phase: 30–55% (v/v) acetonitrile in ammonium acetate buffer (pH 6) (0.050 mol/l).

Compound	Acetonitrile (%)	Capacity factor		α	Capacity factor		α
		X_1	X_2		D_1	D_2	
Atenolol	30	5.36	7.18	1.34	19.77	27.74	1.40
Metoprolol	45	6.32	8.38	1.33	15.95	21.64	1.36
Propranolol	55	5.25	6.42	1.22	10.05	12.86	1.28
Alprenolol	55	6.05	6.92	1.14	11.20	14.03	1.25

X_2 was small. When the added concentration of metoprolol was lowered from 10 to 0.10 mmol/l, the amount of derivatives X_1 and X_2 decreased only slightly, whereas the amount of derivatives D_1 and D_2 decreased more than in proportion to the added metoprolol concentration, so that a straight line fitted to these data had an intercept at about 0.05 mmol/l (Fig. 3). At even lower levels, the amount of X_1 and X_2 formed tended to be proportional to metoprolol, whereas the slope of the D_1 and D_2 curves was lower (*i.e.*, the yield was lower) than at higher levels.

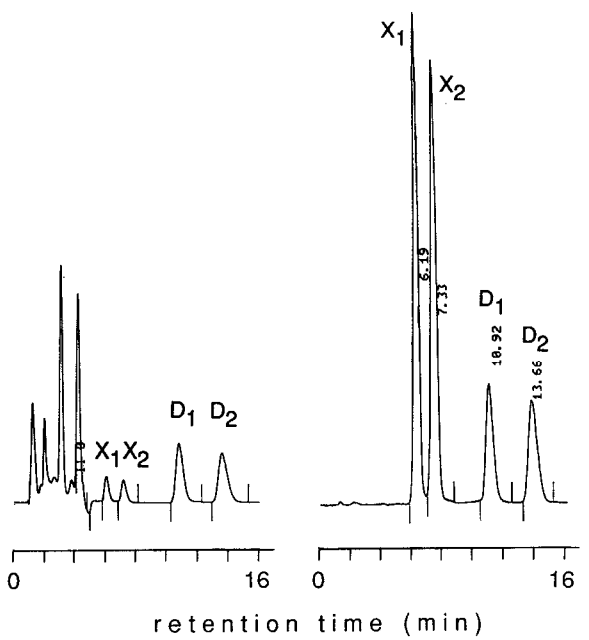

Fig. 2. Separation of products of (*S*)- and (*R*)-propranolol reacted with GITC (0.56 m*M* propranolol racemate, 4 m*M* GITC and 110 m*M* triethylamine in acetonitrile, 20 min at room temperature). Chromatographic conditions as in Table I. Flow-rate, 1.0 ml/min. Left, UV detection (228 nm); right, fluorescence detection (excitation at 228 nm, emission at 342 nm).

Further, when the added metoprolol concentration was held constant at 0.40 mmol/l and the GITC concentration was increased from 10 to 40 mmol/l (25–100-fold molar excess), the amount of X_1 and X_2 formed increased. The same experiment with 0.040 mmol/l of metoprolol (250–1000-fold molar excess of GITC) gave a more or less constant amount of X_1 and X_2 (Fig. 4).

The findings can be explained by the presence of a reactive impurity in the GITC reagent. The observation that the amounts of the products X_1 and X_2 follow the GITC concentration except for low levels of metoprolol could then be explained by the limited amount of the impurity, together with its much higher reactivity. Thus the impurity will be depleted by the reaction with metoprolol except for very low levels of the latter.

Determination of the amount of reactive impurity in GITC

UV chromatograms of the GITC reagent, recorded using the conditions in Table I (45% acetonitrile), were of little value for detecting the impurity. Preparative chromatography of the impurity was considered difficult also because of its reactivity. Therefore, its presence and properties had to be measured indirectly, via the formation of the products X_1 and X_2.

For the first estimation we make the assumption that X and D are the only reaction products and,

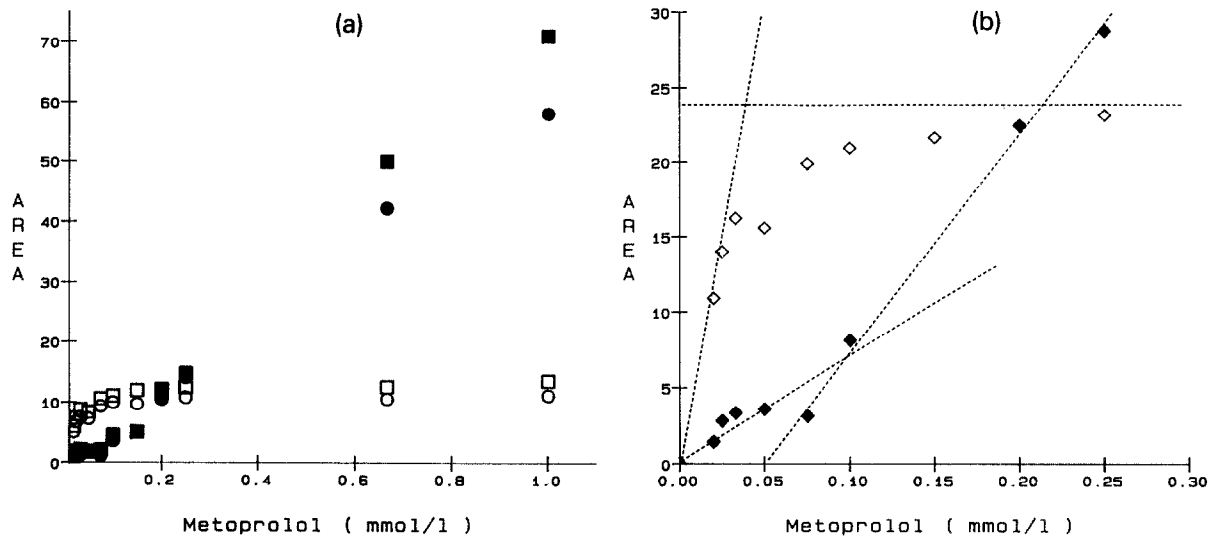

Fig. 3. (a) Amounts of products X and D, measured as fluorescence peak areas, obtained from various amounts of metoprolol racemate (added concentration 0.01–1.0 mmol/l) after rection with GITC (10 mmol/l). ○ = Peak X_1 [by-product from (*S*)-metoprolol]; □ = peak X_2 [by-product from (*R*)-metoprolol]; ● = peak D_1 [main product from (*S*)-metoprolol]; ■ = D_2 [main product from (*R*)-metoprolol]. (b) Expanded plot for low concentrations of metoprolol showing peak areas for (◇) $X_1 + X_2$ and (◆) $D_1 + D_2$. Fluorescence excitation and emission wavelengths, 228 and 306 nm, respectively. Chromatographic conditions as in Table I.

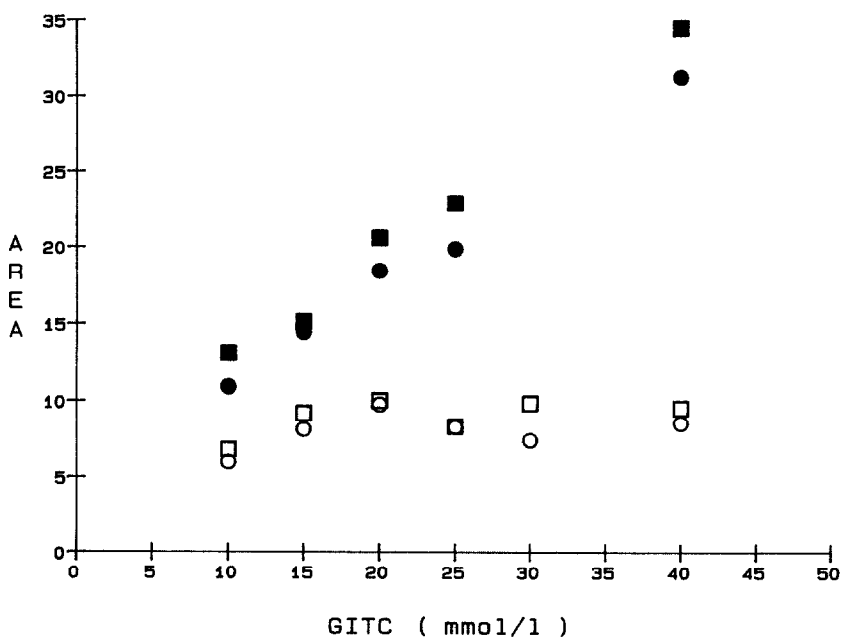

Fig. 4. Amounts of by-product X, measured as fluorescence peak area, obtained from metoprolol racemate after reaction with various amounts of GITC (10–40 mmol/l). (○) (*S*)- and (□) (*R*)-metoprolol 0.04 mmol/l; (●) (*S*)- and (■) (*R*)-metoprolol 0.40 mmol/l.

consequently, the sum of their yields is quantitative as long as no unreacted metoprolol is detected. Then the more or less constant level of X for higher concentrations of metoprolol correspond to a loss (z_0) of the main product D, so that peak heights for D (y) *versus* metoprolol concentration (z) follow the relationship.

$$y = k(z - z_0)$$

In Fig. 3, the intercept z_0 can be estimated to be 0.050 ± 0.025 mmol/l. Hence the original concentration of the reactive impurity in GITC would be about 0.050 mmol/l, which is 0.5% of the added GITC concentration, 10 mmol/l.

A second estimation of the amount of reactive impurity can be made from results for low levels of metoprolol, below 0.050 mmol/l. Here, the slopes of the area *versus* concentration curves for X and D as they approach the origin can be estimated. The slope for D is roughly half of the slope for higher concentrations, corresponding to a recovery of about 50% for D. This would imply that the recovery of X at low levels also is about 50% of added metoprolol. The difference in slope for X and D reflects the difference in molar fluorescence response, which would then be about eight times higher for the products X. Now, the constant level of X for high concentrations of added metoprolol can be quantified by dividing peak areas for X by eight and comparing them with the response for D at high levels of added metoprolol. This gives a value of about 0.025 mmol/l, which is lower but still of the same order as the first estimate. To explain why the recoveries of the products X and D are both about 50% at low levels of metoprolol, the impurity must be over two orders of magnitude more reactive than GITC itself under the reaction conditions used.

Avoiding formation of by-products X

It should be possible to remove a small but highly reactive impurity by adding an amount of another amine to the reagent prior to derivatization. N-Isopropylamino-2-propanol (H 170/37) was chosen to check this. When 1 mmol/l of this amine was added to 10 mmol/l of GITC 1 min before metoprolol was allowed to react, only the products D_1 and D_2 were detected (Fig. 5), which is in accordance with our assumption.

Mass spectrometric investigation of reaction products

Propranolol was chosen for liquid chromatographic–mass spectrometric investigations owing to more favourable chromatographic separation of the derivatives from other sample components, especially GITC. The mass spectral fragmentation pattern was also more favourable than for metoprolol and its derivatives. The products D_1 and D_2 gave similar positive-ion mass spectra, one of which is shown in Fig. 6. A molecular ion was not observed, but the ions at m/z 260 and 407, which also are present in the mass spectra of propranolol (M + 1) and GITC (M + 18) confirmed that these peaks were from the thiourea derivatives of GITC and propranolol. X_1 and X_2 gave positive ions at m/z 633 and negative ions at m/z 631, suggesting a molecular weight of 632, which is 16 mass units lower than that of the proper GITC derivatives. A positive ion at m/z 260 showed that the propranolol fragment was present.

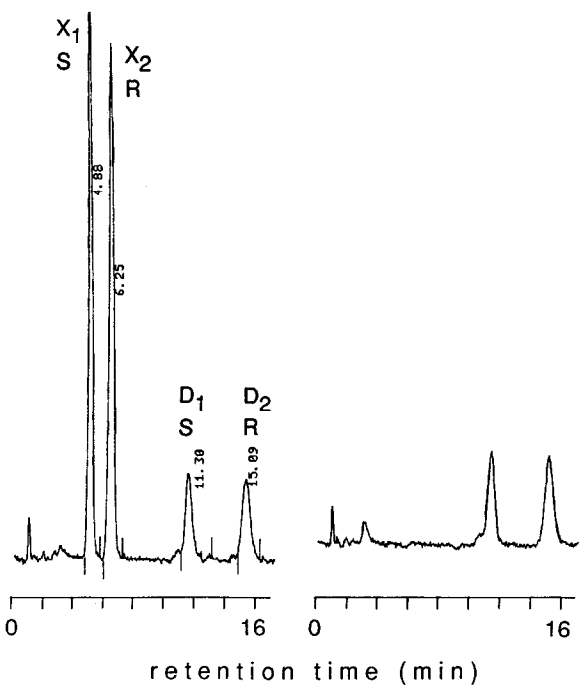

Fig. 5. Separation and fluorescence detection of metoprolol racemate reacted with GITC (0.98 m*M* metoprolol, 9.8 m*M* GITC and 10.9 m*M* triethylamine in acetonitrile). Right: 1.0 m*M* amine (H 170/37) was added to the reaction mixture 1–2 min before metoprolol was added. Excitation at 228 nm, emission at 306 nm. Chromatographic conditions as in Table I.

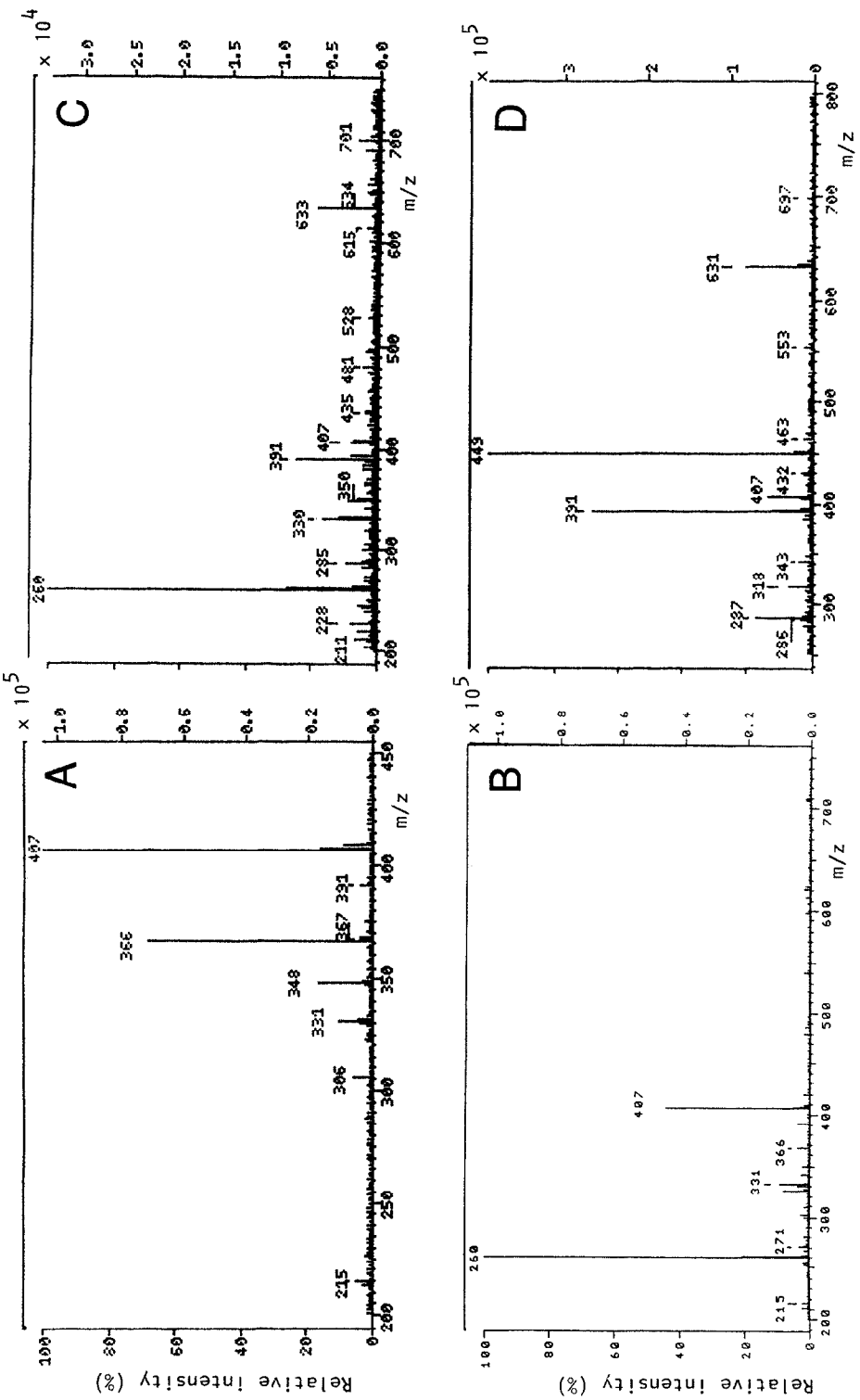

Fig. 6. Thermospray mass spectra of GITC and reaction products with propranolol. Samples (100 μl) of GITC solution (0.50 mmol/l) or reaction mixture (0.17 m*M* propranolol, 6 m*M* GITC and 82 m*M* triethylamine) were separated on an octadecylsilica column (see Table I and Experimental for details). (A) GITC, positive ions; (B) product D_1, positive ions; (C) product X_2, positive ions; (D) product X_2, negative ions. Right hand scales indicate absolute ion intensities (arbitrary units).

Testing possible GITC contaminants

Two substances were considered as possible contaminants of GITC, namely the corresponding bromide and isocyanate. The bromide was tested as a reagent but was found, as could be expected, not to react with β-blockers under the given experimental conditions. The isocyanate was produced from the bromide by reaction with silver cyanate, in analogy with the synthesis of GITC [3]. It was found to react faster than GITC with metoprolol. The diastereomeric derivatives eluted earlier than the GITC derivatives and close to the derivatives X_1 and X_2. They also exhibited fluorescence of similar intensity to X_1 and X_2, but they were eluted as a single peak instead of two separate peaks, and therefore could not be identical with X_1 and X_2. Further, the isocyanate had limited stability in acetonitrile, as opposed to the reactive impurity in GITC.

Reactive impurity in α-methylbenzyl isothiocyanate

α-Methylbenzyl isothiocyanate was studied to see whether a similar competing reaction occurred. When propranolol racemate was allowed to react with this reagent, a pair of peaks was observed and, in addition, one peak with a retention roughly half that of the other two peaks. This peak disappeared when the reagent (13.5 mmol/l) was treated with an amine (H 170/37, 1.7 mmol/l) before propranolol was added. When the isothiocyanate reagent was replaced with the corresponding isocyanate, propranolol racemate gave one peak instead of three. Its retention was virtually the same as that for the early-eluting peak from the isothiocyanate reagent. Also, the fluorescence yield was similar. A chromatogram of the isothiocyanate reagent alone showed a small peak which coincided with that of the isocyanate. However, no final proof was obtained for the identity of the impurity in the α-methylbenzyl isothiocyanate reagent.

CONCLUSION

Derivatization of β-blockers with commercial or in-house prepared GITC and separation of the reaction products gives two pair of peaks, one pair originating from the expected diastereomeric thiourea derivatives. The products seen as the other two peaks appear to be closely related to the thiourea derivatives, but remain to be identified. With a large excess (250-fold or more) of the reagent, the yield of both types of derivatives was similar, about 50%. With a decreasing excess of reagent, the relative yield of the unidentified products decreased, favouring the formation of the proper thiourea derivatives. The formation of the unidentified products can be avoided by treating the reagent with another amine prior to derivatization. The unidentified products exhibit stronger fluorescence than the thiourea derivatives, which would make them attractive for analytical applications. Identification of their chemical structure might lead to a new chiral reagent that could be an interesting alternative to GITC.

ACKNOWLEDGEMENT

The mass spectral recordings were carried out by Karl-Erik Karlsson, Analytical Chemistry, Astra Hässle.

REFERENCES

1 P. Edman, *Acta Chem. Scand.*, 4 (1950) 283.
2 T. Nambara, S. Ikegawa, M. Hasegawa and J. Goto, *Anal. Chim. Acta*, 101 (1978) 111.
3 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
4 N. Nimura, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 213 (1981) 327.
5 T. Walle, D. D. Christ, U. K. Walle and M. J. Wilson, *J. Chromatogr.*, 341 (1985) 213.
6 J. Gal, *J. Liq. Chromatogr.*, 9 (1990) 673.
7 J. Gal and A. J. Sedman, *J. Chromatogr.*, 314 (1984) 275.
8 J. Gal, D. M. Desai and S. Meyer-Lehnert, *Chirality*, 2 (1990) 43.
9 J. S. Davies and A. K. A. Mohammed, *J. Chem. Soc., Perkin Trans. 2*, (1984) 1723.
10 D. Schuster, M. Woodruff Modi, D. Lalka and F. M. Gengo, *J. Chromatogr.*, 433 (1988) 318.
11 E. J. Eisenberg, W. R. Patterson and G. C. Kahn, *J. Chromatogr.*, 493 (1989) 105.
12 S. E. Rose and E. J. Randinitis, *Pharm. Res.*, 8 (1991) 758.