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GAS CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION OF METOPROLOL FROM HUMAN PLASMA AFTER REACTION WITH PHOSGENE

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SUMMARY

A method for the determination of therapeutic levels of metoprolol in human plasma is presented. Metoprolol and the internal standard are extracted from the buffered plasma sample to an organic phase containing 4×10^{-3} *M* phosgene. After 10 min the organic phase is taken to dryness. The residue is dissolved in ethyl acetate and the formed oxazolidine derivatives are analyzed by gas chromatography with nitrogen-selective detection.

With packed columns, rectilinear standard curves through the origin were obtained down to 80 nmoles/l of plasma. The precision of the method at 200 nmoles/l was 1.5% (n = 8).

The sensitivity of the method was improved by using capillary column gas chromatography. Linear standard curves were obtained down to 10 nmoles/l of metoprolol in plasma. The precision of the method at the 50 nmoles/l level was 2.2% (n = 7).

With this simple and straightforward method using extractive derivatization 30 samples can be handled in a day.

INTRODUCTION

In addition to pharmacokinetic studies, the monitoring of plasma levels of β-blocking drugs is of a certain importance in drug compliance and toxicological cases. The most common method used is gas chromatography with electron-capture detection of perfluoroacyl derivatives [1, 2]. In most instances it is possible to perform the derivatization after a simple extraction without any extra purification steps. However, due care has to be taken to ensure reliable derivatization. The quality of the perfluoroacyl reagents may vary from batch to batch, sometimes resulting in low yield or extraneous peaks. Besides, the risk of hydrolysis can never be ruled out completely. Recently, it has been demonstrated that this type of derivative exhibits instability on certain capillary columns [3].

Phosgene is widely used in industry for the production of a great number of

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chemical compounds in large quantities. It is commonly used in organic synthesis, normally under anhydrous conditions [4—6]. The use of phosgene as a derivatizing agent in gas chromatographic analysis is rare [7]. A reverse procedure exists [8], however, for the trapping and determination of phosgene formed metabolically from chloroform [9] and carbon tetrachloride [10] with cysteine. The formed oxathiazolidine carboxylic acid is isolated and methylated with diazomethane prior to quantitation by gas chromatography—mass spectrometry.

Our interest in phosgene as a potential derivatizing agent for amino alcohols originated from the use of chloroformates in aqueous media [11, 12] and the use of boronates as cyclization reagents [13]. As phosgene is slowly hydrolyzed by water [14, 15] it looked promising as a derivatizing agent in an aqueous environment.

In the present method, for the β-blocking drug metoprolol in plasma, extraction and derivatization with phosgene are performed simultaneously. After evaporation of the organic phase the residue is analyzed by gas chromatography with nitrogen-selective detection.

EXPERIMENTAL

Gas chromatography

A Varian 3700 gas chromatograph with flame ionization and thermionic (nitrogen—phosphorus-selective) detectors was used equipped with 120 × 0.2 cm I.D. glass columns. These were filled with 3% OV-17 on 100—120 mesh Gas-Chrom Q or 3% Hi-EFF-8BP (= cyclohexanedimethanol succinate) on the same support. Gas flow-rates of nitrogen carrier gas were 30 or 45 ml/min. The thermionic detector was supplied with 5 ml of hydrogen and 175 ml of air per min. The detector bead current setting was in the range 400—500. The injector, oven and detector were maintained at 250°C, 240°C and 300°C, respectively.

Capillary column gas chromatography was performed in the same instrument by the aid of stainless-steel adaptors for split effluent and make-up gas [16]. A soda glass column (7 m × 0.26 mm I.D.) coated dynamically with Carbowax 20M was used [17]. The inlet pressure of helium carrier gas was 150 kPa. The carrier gas flow-rate was 7 ml/min and that of the split effluent 20 ml/min (both measured at 150°C). The flow-rate of helium make-up gas was 20 ml/min. The temperature of the injector and the detector were as above. Samples were injected with the split valve closed at a column temperature of 150°C. After 0.8 min the split valve was opened and after 1 min the column temperature was increased to 240°C at 30°C/min.

Mass spectrometry

A Varian MAT-112 mass spectrometer coupled to a Varian 1400 gas chromatograph was used. The glass column was filled with the same OV-17 packing as above. Helium was used as carrier gas at a flow-rate of 20 ml/min. After injection and venting of the solvent, the slit valve to the ion source was opened manually to a pressure of 2×10^{-5} Torr. The temperatures were 260°C (oven) and 250°C (injector, transfer line and ion source). The electron energy was

70 eV and the emission current 1.5 mA.

Liquid chromatography

The system consisted of a pump (Altex 110A), an injection valve (Rheodyne 7010) fitted with a sample loop (250 μl), a 150 × 4.5 mm stainless-steel column filled with 5 μm LiChrosorb RP-8 and a Cecil 212 variable-wavelength ultraviolet (UV) monitor. The mobile phase was 0.01 *M* phosphoric acid in 60% methanol (metoprolol oxazolidine) and 0.01 *M* of tetrabutylammonium hydrogen sulphate in aqueous buffer pH 2 (μ = 0.1) with 9% of acetonitrile (metoprolol). The flow-rate was 1 ml/min and the UV absorption of the eluate was measured at 276 nm.

Liquid scintillation

The radioactivity measurements of the fractions containing radioactive metoprolol and derivative from the liquid chromatography eluate were performed by liquid scintillation counting. Insta-Gel^{R} (Packard) was added to the samples before counting on a Mark III spectrometer (Searle Analytical Instruments). The quenching was corrected for by external standardization.

Reagents and chemicals

Phosgene 2 *M* in toluene, purum, was from Fluka (Buchs, Switzerland), methylene chloride and ethyl acetate p.a. from Merck (Darmstadt, G.F.R.), acetonitrile and hexane HPLC grades were from Rathburn Chemicals (Walkerburn, Great Britain).

Metoprolol, (1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy-2-propanol]-tartrate), H 87/31 (internal standard, 2-ethoxymethyl), H 93/47 (internal standard for electron-capture gas chromatography, ethoxyethyl) and tetrabutylammonium hydrogen sulphate were synthesized at AB Hässle (Department of Organic Chemistry).

3-Isopropyl-5-[4-(2-methoxyethyl)phenoxymethyl] oxazolidine-2-one, the oxazolidine derivative of metoprolol, was synthesized from metoprolol base dissolved in pyridine and a one-fold excess of phosgene in toluene. After standing overnight, excess phosgene was destroyed by the addition of water. The toluene phase was washed twice with 1 *M* hydrochloric acid before evaporation. The crystalline residue was recrystallized with boiling isopropyl ether. The m.p. was 81—82° C. Büchi 2° C/min.

Tritiated metoprolol (AB Hässle) was at a concentration of 2.5 μM in distilled water (250,000 dpm/ml).

n-Octacosane from Fluka and trichloroethyl carbamate of dibenzylamine [11] were used as non-reacting internal standards.

Sodium phosphates and carbonates were used for the preparation of aqueous buffers (μ = 1).

METHODS

Determination of metoprolol in plasma

Plasma (2 ml or 2 g) and 1 ml of buffer pH 8.1 with 900 n*M* of the internal standard H 87/31, 5 ml of the extraction solvent (hexane—methylene chloride,

4:1) and 10 μl of 2 *M* phosgene in toluene were shaken horizontally for 10 min (160 rpm). After centrifugation the organic phase was transferred to a 5-ml centrifuge tube, either after freezing of the aqueous phase (dry ice and acetone) or by using a pipette. The organic solvent was removed by evaporation with a stream of nitrogen. The residue was dissolved in 25—50 μl of ethyl acetate and 1—2 μl was taken for analysis by gas chromatography on 3% Hi-EFF-8BP with nitrogen-selective detection.

Samples for analysis with capillary column gas chromatography were prepared by the same procedure except that 1 ml of plasma was taken and that the concentration of the internal standard was 225 n*M*. Of the final 25-μl solution 1 μl was injected into the gas chromatograph equipped with a Carbowax 20M capillary column and nitrogen-selective detection.

Electron-capture gas chromatography of metoprolol from plasma

Plasma (2 g), 0.3 ml of 1 *M* sodium hydroxide and 100 μl of the internal standard, 7.29 μ*M* (H 93/47), were extracted for 10 min with 5 ml of hexane—methylene chloride (4:1). After centrifugation and freezing of the aqueous phase the organic phase was decanted to a new tube and mixed with 200 μl of trifluoroacetic anhydride. The reaction was allowed to proceed for at least 45 min at 40°C before evaporation with nitrogen. The residue was dissolved in 200 μl of toluene and a 3-μl aliquot was analyzed by gas chromatography on 3% OV-17.

RESULTS AND DISCUSSION

Identity of derivative formed

The derivative formed by the reaction of metoprolol with phosgene was identified by gas chromatography—mass spectrometry. The structure and the mass spectrum are presented in Fig. 1. Prominent ions are *m/z* 100 and 56. The former is formed by the loss of isopropyl from *m/z* 142 which is followed by the elimination of carbon dioxide to give *m/z* 56. The latter ion is also dominant in the fragmentation of cyclic boronates of alprenolol [18]. The ions

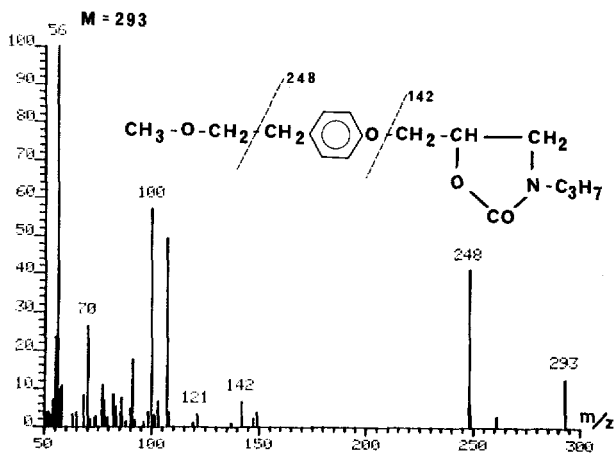

Fig. 1. Mass spectrum of the phosgene derivative of metoprolol at 70 eV.

in the higher mass range have sufficient relative intensity to be promising for mass fragmentography.

Reaction conditions

pH of the aqueous phase. Initially the derivatization reaction with phosgene was carried out in aqueous buffer without plasma or organic phase present. A constant yield of the derivative was obtained in the pH range 7.5—12.1 as shown in Table I. A buffer of pH 8.1 was selected as a suitable medium for the reaction.

TABLE I

INFLUENCE OF pH ON THE DERIVATIZATION OF METOPROLOL WITH PHOSGENE IN AQUEOUS MEDIA

Method: 1 ml of 1 mg/ml metoprolol in 0.01 *M* hydrochloric acid was mixed with 1 ml of buffer (μ = 1) and the pH was measured. Then 50 μl of 2 *M* phosgene in toluene were added and the mixture was vibrated for 30 sec and allowed to stand for 5 min before extraction with 5 ml of methylene chloride containing 1 mg of *n*-octacosane. Then 0.5 ml of the organic phase was taken to dryness and dissolved in 50 μl of ethyl acetate before analysis by gas chromatography with flame ionization detection.

Starting pH	Relative yield (%)	Final pH
6.2	28	4.9
6.6	69	6.0
7.0	87	6.2
7.5	99	6.5
8.1	103	6.6
8.3	101	6.7
9.7	93	7.7
10.4	98	9.5
12.1	99	11.1

Presence of plasma. When plasma was present in the reaction mixture the yield of the derivative was reduced relative to that obtained when buffer only was the reaction medium. This may be due to the inclusion of metoprolol or the derivative in the protein precipitate formed by the rather vigorous shaking conditions used at this stage. The problem could be circumvented if the extraction solvent was present before the addition of phosgene. The yield of the derivative was then the same as the aqueous reference. An advantage with this procedure is also that less handling of the sample is required when derivatization and extraction are performed simultaneously, so called extractive derivatization (cf. extractive alkylation, acylation or dealkylation).

Partition properties of the metoprolol derivative. The distribution ratio of the oxazolidine derivative of metoprolol is independent of pH. It was found to be 2.3 in the system hexane—buffer pH 8.1 (μ = 1) with equal phase volumes. For the intact compound this ratio is 1.3 at pH 11 (pK_a + 1.3, negligible amounts of ionized metoprolol present). This means that only a slight increase in the lipophilic character is obtained by derivatization with phosgene. The

practical implication of this is that only 92% of the derivative will be recovered in the hexane phase if a phase ratio of 5:1 is used. By including 20% of methylene chloride in the hexane phase less than 1% of the derivative could be retrieved from the aqueous phase when this was subjected to a new extraction.

Amount of phosgene. The amount of phosgene required for a constant yield in the two-phase system at pH 8.1 is illustrated in Table II. The yield of derivative is constant from 5 to 20 μl of 2 *M* phosgene added; 10 μl was selected as a suitable amount. The molar excess of phosgene was approximately 10 in this study (C_0 metoprolol 8×10^{-4} *M*). At lower concentrations of the substrate the excess of the reagent will be considerable. Plasma constituted 25% of the aqueous phase in these experiments and appears to have no detrimental effect on the yield when compared with the aqueous reference samples (Table II). It was later found that plasma up to 67% of the aqueous phase had no harmful effect on the yield (0.4 μmoles/l of metoprolol in plasma).

Internal standard. An analogue of metoprolol, H 87/31, was selected as a suitable internal standard. The only structural difference is that of the position of the ether oxygen in the short side-chain. With 3% Hi-EFF-8BP as stationary phase it elutes just before metoprolol (Fig. 2).

Reaction time. When the time course for the reaction of phosgene with metoprolol and the internal standard was followed in the two-phase system with plasma present, a constant peak height ratio was obtained from 5 min up to 2 h. A shaking time of 10 min was used hence-forward.

TABLE II

AMOUNT OF PHOSGENE REQUIRED FOR THE DERIVATIZATION OF METOPROLOL IN A TWO-PHASE SYSTEM WITH PLASMA PRESENT

Method: 0.5 ml of buffer pH 8.1, 1 ml of metoprolol 0.46 mg/ml in 0.01 *M* hydrochloric acid and 0.5 ml of plasma were mixed and shaken together with 5 ml of hexane—methylene chloride (4:1) and phosgene as indicated in the table. The organic phase also contained 106 μg/ml of the trichloroethyl carbamate of dibenzylamine as marker. After 5 min shaking a 0.5-ml aliquot was withdrawn and evaporated. The residue was dissolved in 50 μl of ethyl acetate and analysed by gas chromatography with flame ionization detection.

Amount of 2 *M* phosgene in toluene added (μl)	Relative yield (%)
20	100 ± 4*
20	98
20	99
15	97
15	98
10	97
10	98
5	97
5	100
2	88
2	82
1	78
1	72

*Aqueous reference, $n = 3$.

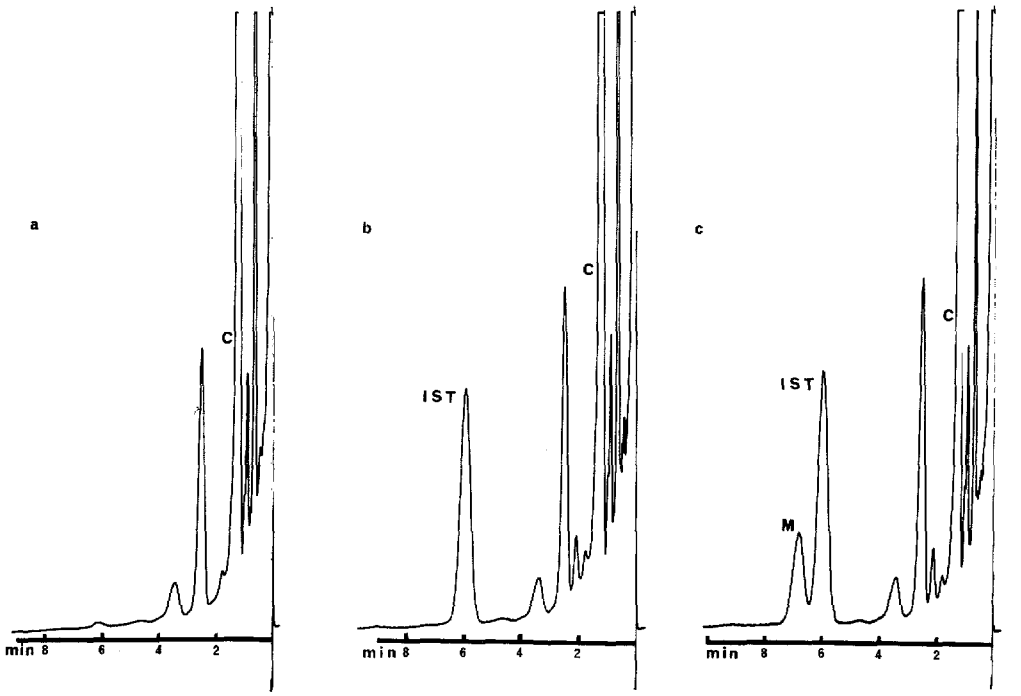

Fig. 2. Packed column gas chromatograms from the analysis of metoprolol in plasma. (a) Blank plasma. (b) Blank plasma and 900 pmoles of the internal standard. (c) As in (b) plus 200 nmoles/l of metoprolol. Column: 3% cyclohexanedimethanol succinate at 240°C; 2 μl injected (of 25 μl final volume) at attenuation 8. M = metoprolol, IST = internal standard, C = caffeine.

Stability of phosgene and the metoprolol derivative. In the presence of water phosgene is hydrolyzed to hydrogen chloride and carbon dioxide. The hydrolysis of phosgene in the two-phase system used was studied in the absence of metoprolol. At various time intervals aliquots of the organic phase were withdrawn and mixed with an excess of metoprolol [19] and a known amount of the trichloroethyl carbamate of dibenzylamine as marker. The amount of derivative formed was taken as a measure of the amount of phosgene present. Without plasma present the half-life of phosgene was 4 min and with plasma present ca. 2 min. As the extrapolated initial concentration of phosgene from the plot coincided with that of the initial concentration before the aqueous phase was added, the conclusion is that phosgene is mainly present in the organic phase. From the calculated half-life with plasma present, 2 min, phosgene can be expected to be present in ca. 3% of the initial concentration after 10 min. The corresponding amount is equal to the threshold limit value for phosgene if the organic phase is completely evaporated in 100 l of air. This fact emphasizes that the sample tubes should be handled in a vented hood, and that the reaction time should not be reduced, in order to prevent the risk of exposure to phosgene.

The stability of the oxazolidine derivatives was investigated shaking equal phase volumes of organic phase and 0.1 *M* of sulphuric acid or sodium hydroxide as the aqueous phase. The initial concentration of the derivatives

was 1 μM and a suitable amount of the trichloroethyl carbamate of dibenzylamine was added as marker. At various time intervals aliquots were withdrawn and analysed by gas chromatography after evaporation and reconstitution in a small volume of ethyl acetate. No degradation of the derivatives could be observed for up to 8 h.

Standard curves, precision and absolute recovery

The reaction conditions were optimized using milligram amounts of metoprolol and gas chromatographic analysis with flame ionization detection and OV-17 as stationary phase. When the amounts injected were reduced a hundredfold, and the nitrogen-selective detector was used, severe peak broadening was noted. Some more polar silicone stationary phases were tested but without success (QF-1, OV-225 and Silar 5 CP). The peak symmetry on the cyanosilicone phases was adequate but the bleeding excluded the use of these phases in combination with the nitrogen-selective detector. The polyester phase cyclohexanedimethanol succinate (Hi-EFF-8BP) proved to be suitable for the analysis of nanogram amounts of the oxazolidine derivatives on column. Symmetrical peaks (Fig. 2) and acceptable precision was obtained when 5-ng amounts were injected repeatedly and quantitated with an inert internal standard (S.D. $< 3\%$, $n = 6$).

Linear standard curves obtained from 800 to 80 nmoles/l of plasma (213—21 ng/ml) passed through the origin. The precision at the 200 nmoles/l level was 1.5% ($n = 8$).

The absolute recovery of metoprolol as oxazolidine derivative in the organic phase was 85% as determined with the synthesized material as reference. This value was supported by liquid chromatography and liquid scintillation counting.

Selectivity of the present method

Actual plasma samples from patients on metoprolol therapy were analysed by the present method and the electron capture gas chromatography method [2] used for routine determinations of metoprolol in our laboratories. The results of the comparison are shown in Fig. 3. The latter method tends to give somewhat higher values.

Interferene from metoprolol metabolites is not likely. The major metabolites have alcoholic hydroxyl groups beside the intact β-sidechain [20] or the β-sidechain oxidized to a carboxylic group [20]. The alcoholic metabolites are less probable to interfere as they require further derivatization prior to gas chromatography, e.g. trimethylsilylation of the alcohol group [21]. The carboxylic acid metabolite is not likely to cyclize with phosgene under the present conditions.

The secondary amine 1-isopropylamino-3-[4-(2-methoxyethyl)phenoxy]-propane (metoprolol devoid of the alcohol group) does also react with phosgene. The formed chlorocarbonyl derivative (identified by mass spectrometry) could only be chromatographed at low levels after treatment with methanol, that is after conversion to its methyl carbamate derivative. Thus, interference from moderate levels of amines is not likely either. This shows clearly the advantage of selective cyclic derivative formation [7].

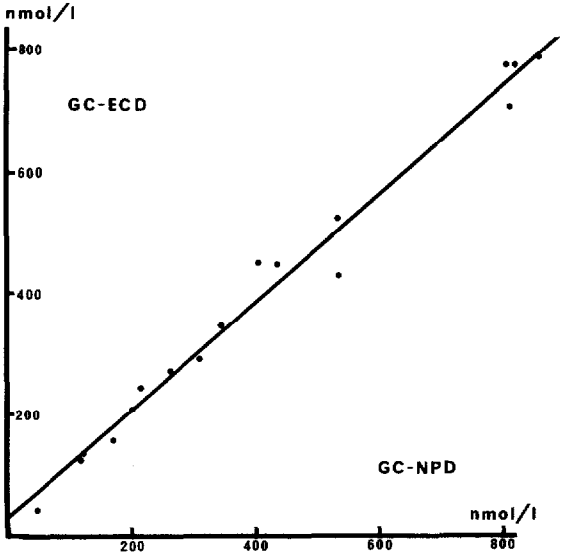

Fig. 3. Comparison between the trifluoroacetylation method and the phosgene derivatization method for metoprolol. GC—ECD = gas chromatography with electron-capture detection after trifluoroacetylation; GC—NPD = gas chromatography with nitrogen-selective detection after reaction with phosgene. Linear regression = 0.991, slope = 0.877, intercept = 31 nmoles/l, number of samples = 17.

The present derivative of metoprolol has a five-membered ring structure. Also, six- and seven-membered rings can be formed and chromatographed as was demonstrated with two metoprolol homologues with one or two extra methylene groups inserted between the amino and the hydroxy group [3], identified by mass spectrometry.

Derivatization of some other β-blocking drugs

The relative retention of some other β-blocking drugs after phosgene derivatization is shown in Table III. The table shows the potential of this method for the determination of other drugs of this type. Some of the more structurally complex β-blocking drugs gave two peaks in the chromatograms, e.g. pamatolol and timolol. The poor symmetry of the atenolol peak could be improved by derivatization of the amide moiety to the corresponding nitrile by trifluoroacetic anhydride [22]. The derivatization and extraction conditions were not optimized in any of these experiments. Gas chromatography with mass spectrometry was used to confirm the structures of the formed derivatives.

Interferene from caffeine

Some plasma samples from patients contained high concentrations of caffeine (> 1 μg/ml). This resulted in an overload of the chromatographic system, as confirmed by injecting corresponding high amounts of pure caffeine. In the present chromatographic system this interference did not affect the derivative peaks. The position of caffeine in a normal chromatogram is indicated in Fig. 2. With the actual organic phase less than 10% of the caffeine present in the aqueous phase is extracted to the organic phase.

TABLE III

RELATIVE RETENTION OF SOME β-BLOCKING DRUGS AS OXAZOLIDINE DERIVATIVES

Compound	Relative retention time on	
	3% OV-17	3% Hi-EFF-8BP
Alprenolol	0.47	0.40
Oxprenolol	0.64	0.56
H 87/31 (internal standard)	—	0.87
Metoprolol	1.00*	1.00**
Propranolol	1.67	2.12
Pindolol	2.83	7.42

*Absolute retention time: 1.8 min at 240°C.
**Absolute retention time: 6.8 min at 240°C.

Application to capillary column gas chromatography

The sensitivity of the present method was improved by using capillary column gas chromatography. The oxazolidine derivatives chromatographed without any sign of decomposition or adsorption. A chromatogram with 9 ng of the metoprolol derivative is presented in Fig. 4a. The Carbowax 20M column

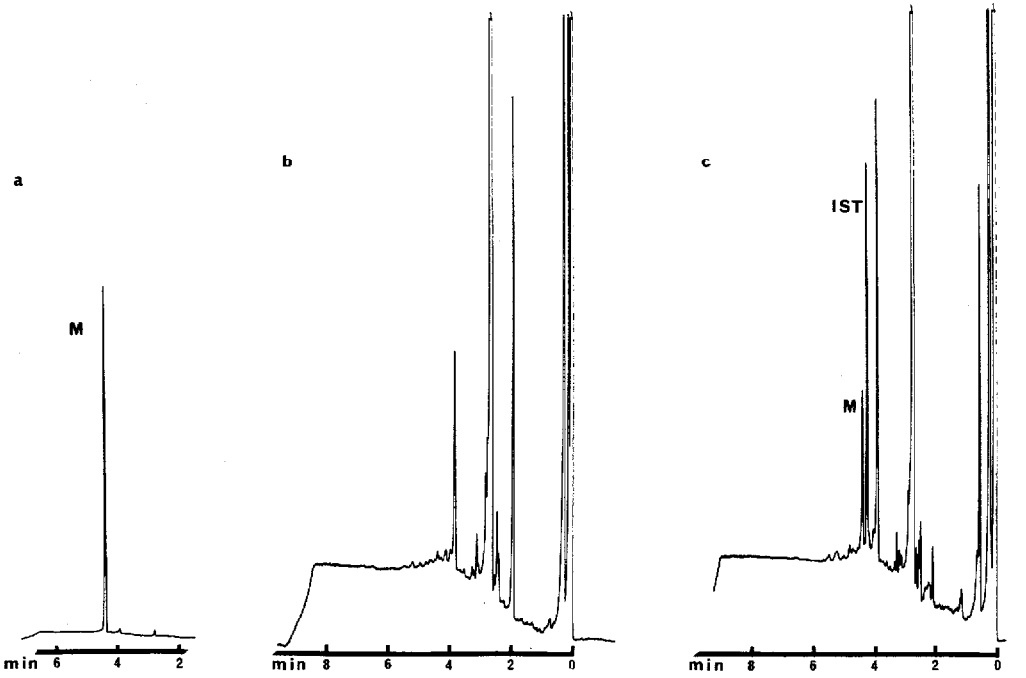

Fig. 4. Capillary column chromatograms from the analysis of metoprolol in plasma. (a) 9 ng of metoprolol derivative, attenuation 64. (b) Blank plasma, attenuation 8. (c) as (b) with 225 pmoles of the internal standard and 100 nmoles/l metoprolol, attenuation 8. Column: Carbowax 20M, 150°C for 1 min then 30°C/min to 240°C; 1 μl injected of the 25 μl final solution (b and c). M = metoprolol, IST = internal standard.

used in this study was not the only column capable of chromatographing these derivatives. A 30 m × 0.25 mm SE-30 fused silica column (DB-1, J and W Scientific) was also used without problem. This indicates an improved stability of these derivatives as compared with perfluoroacylated derivatives on capillary columns [3].

Linear standard curves for metoprolol were obtained down to 10 nmoles/l of plasma. The precision at the 50 nmoles/l level in plasma was 2.2% (n = 7). The presence of minute interfering peaks near that of metoprolol, and not the nitrogen-selective detector, limit the detection of lower levels of metoprolol. Chromatograms from blank plasma and plasma spiked with 100 pmoles of metoprolol and 225 pmoles of the internal standard per 1 ml of plasma are shown in Fig. 4b and c, respectively.

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