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Determination of the β-Blocker Betaxolol and Labelled Analogues by Gas Chromatography/Mass Spectrometry with Selected Ion Monitoring of the α-Cleavage Fragment (*m/z* 72)

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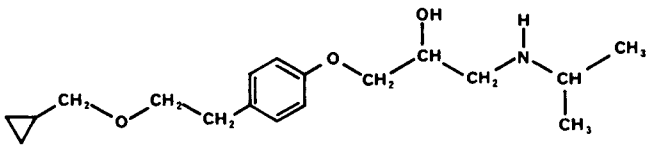
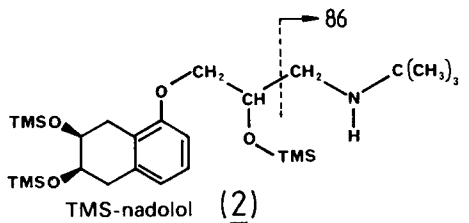
Low concentrations of betaxolol in blood plasma and physiological buffers were determined by selected ion monitoring of the intense *m/z* 72 fragment $[CH_2{=}NH{-}CH(CH_3)_2]^+$ formed by electron impact ionization of the *O*-trimethylsilyl derivative. At a mass spectrometric resolution of 3000, fewer potentially interfering peaks were seen than at low resolution. There remained a chemical interference, corresponding to 100 pg/sample, which arose during treatment of the samples. This method is more sensitive than previous ones, but it is restricted to situations where the specificity can be controlled. When the *m/z* 72 fragment was mass-shifted by using betaxolol appropriately labelled with deuterium or ^{13}C, both the interference and the baseline noise were greatly reduced; concentrations of labelled betaxolol as low as 10–20 pg/sample can be determined with little difficulty.

INTRODUCTION

Routine determinations of betaxolol (**1**) in plasma or tissues have been performed using gas chromatography (GC) with electron-capture detection of the bisheptafluorobutyl derivative,[1–2] and more recently using high-performance liquid chromatography (HPLC) with fluorimetric detection.[3] The limit of useful measurement for both methods is 0.5–1 ng ml^{-1} plasma, and this has been adequate for the majority of clinical investigations. A reference method[4] using gas chromatography/mass spectrometry (GC/MS) with NH_3CI of the *O*-trimethylsilyl (*O*-TMS) derivative provides excellent specificity, but the sensitivity is no better than that of the GC and HPLC methods.



Betaxolol (1)



TMS-nadolol (2)

In the course of some studies on the mode of action of betaxolol *in vivo* and *in vitro*, we required a more sensitive method. When treated with the usual trimethylsilylating reagents, β-blockers having the typical aryloxypropanolamine function are not readily derivatized on the secondary nitrogen. The ion formed by cleavage α to this nitrogen is by far the most intense peak in the electron impact (EI) spectrum (Fig. 1). The TMS derivatives of drugs such as nadolol (**2**) and timolol, which have a *tert*-butylamino group, give *m/z* 86 as the base peak, and sensitive GC/MS methods using low-resolution selected ion monitoring (SIM) of this fragment have been described.[5–7]

This approach does not appear to have been used for compounds which, like betaxolol, have an isopro-

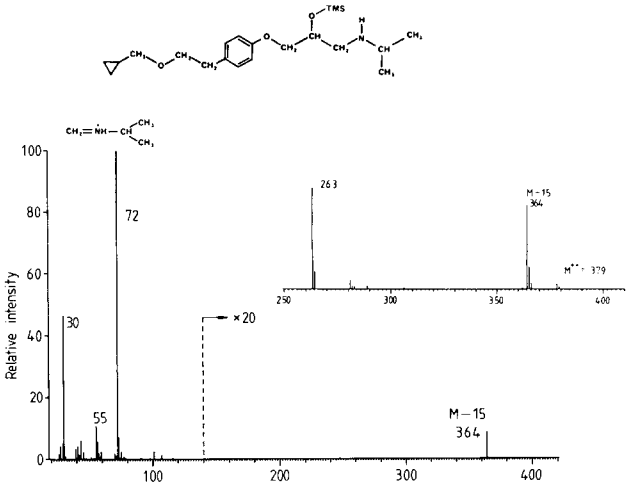


Figure 1. EI mass spectrum of *O*-TMS betaxolol. Inset: part of a spectrum overloaded with respect to *m/z* 72.

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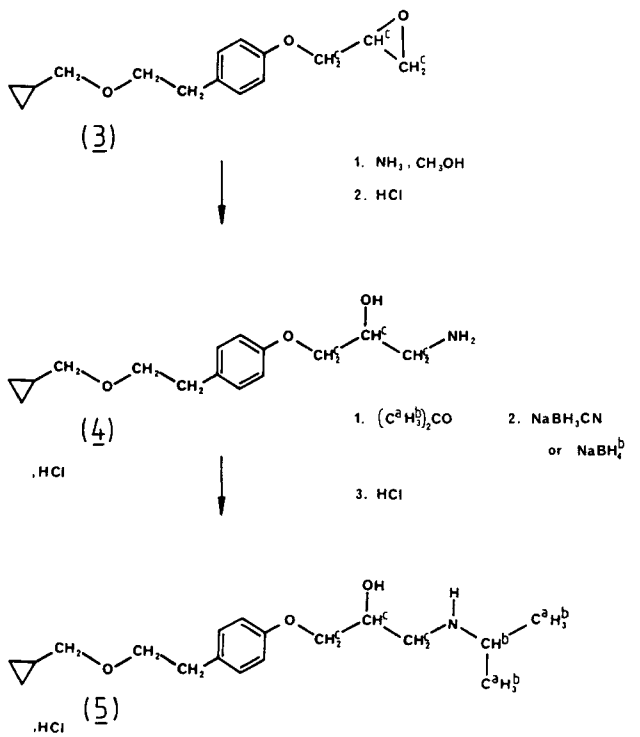
pylamino group. Possibly, the *m/z* 72 fragment is more prone to interference and column bleed than *m/z* 86. For TMS-betaxolol, we have found that much of the interference seen at low resolution can be tuned out, while retaining good sensitivity, by increasing the resolution to 3000. Labelling with a stable isotope gives a mass-shifted α-cleavage fragment virtually free from interference, and labelled betaxolol may be determined down to a few picograms per sample.

MATERIALS AND METHODS

Synthetic methods

Betaxolol and SL 76 020 (a close analogue of betaxolol)[2] were synthesized by the Chemistry Department of LERS.

Three analogues of betaxolol labelled with stable isotopes have been prepared (Scheme 1; Table 1). Details of the synthesis of ($^2H_5$)betaxolol have already been published.[4]



Scheme 1. Synthesis of analogues of betaxolol labelled with deuterium and ^{13}C.

Table 1. Stable isotopically labelled analogues of betaxolol, and *m/z* values of their α-cleavage fragments

Labelling	a	b	c	*m/z*
Unlabelled	^{12}C	^1H	^1H	72.0813
$^{13}C_2$	^{13}C	^1H	^1H	74.0880
$^2H_5$	^{12}C	^1H	^2H	74.0939
$^2H_7$	^{12}C	^2H	^1H	79.1253

1-Amino-3-[4-[2-(cyclopropylmethoxy)ethyl]phenoxy]2-propanol HCl (compound 4). [[4-[2-(Cyclopropylmethoxy) ethyl]phenoxy]methyl]oxirane (**3**)[8] (25 g, 0.1 mol) in methanol (100 ml) was treated with liquid ammonia (50 ml) in a pressure reaction vessel, and the reaction mixture was heated at 100 °C for 16 h. The solvent and excess of ammonia were evaporated, and the product was dissolved in the minimum quantity of methanol, and then treated with an excess of ethereal HCl. The precipitate was collected and recrystallized from methanol/diethyl ether (yield: 22 g; 0.073 mol; 73%; m.p. 130 °C (dec.).

($^{13}C_2$)Betaxolol HCl. Compound **4** (5.1 g, 16.7 mmol) in methanol (40 ml) was treated with methanolic NaOH (0.67 g, 16.7 mmol). ($^{13}C_2$)Acetone (1 g, 16.7 mmol) (CEA, Saclay, France; isotopic purity 90 atom%) was added, and the mixture was stirred at 25 °C for 3h. $NaCNBH_3$ (1.1 g, 16.7 mmol) and glacial acetic acid (1 ml) were then added, and the stirring continued for 1 h. The reaction mixture was evaporated, and the residue treated with dilute HCl, filtered, and the filtrate basified with aqueous ammonia. This was then extracted with CH_2Cl_2 (5 × 50 ml), and the extract washed with water, dried ($MgSO_4$) and evaporated. The residue was purified by chromatography on silica gel, using CH_2Cl_2:MeOH (8:2 v/v) as eluent. The colourless oil thus obtained was dissolved in MeOH, and treated with ethereal HCl. The hydrochloride salt was filtered off and recrystallized from ethanol/ether (yield: 3.7 g, 10.7 mmol, 65%; isotopic purity 91.5%; m.p. 113–114 °C.

($^2H_7$)Betaxolol HCl. Compound **4** (2.71 g, 10.2 mmol) and ($^2H_6$)acetone (Spectrometrie Spin Technique, France; isotopic purity 99.7%) were stirred at 25 °C for 19 h. The excess of acetone was evaporated, and the product dissolved in ($^2H_1$)methanol (CEA) (5 ml). ($^2H_4$)Acetic acid (CEA) (500 μl) and sodium borodeuteride (CEA) (360 mg, 8.6 mmol) were added to the solution with cooling. The mixture was allowed to warm to room temperature, and was stirred for 1 h. The solvent was evaporated, water was added, and the product was extracted with CH_2Cl_2. The combined extracts were washed with water, dried ($MgSO_4$) and evaporated. The residue was purified and converted to the hydrochloride as described above (yield: 1.2 g, 3.4 mmol, 33%; isotopic purity 87%; m.p. 113–114 °C. The following deuterium content (%) was determined by mass spectrometry: $^2H_1$ 0.42, $^2H_2$ 0.44, $^2H_3$ 0.97, $^2H_4$ 3.6, $^2H_5$ 17.6, $^2H_6$ 34.4, $^2H_7$ 42.5.

The structures of the labelled compounds were verified by nuclear magnetic resonance (NMR), infrared (IR) and mass spectrometry.

Solvents and reagents

Acetonitrile and methanol were Merck spectroscopic grade. Diethyl ether (Carlo Erba, Milan, Italy; with stabilizer) was passed through activated alumina (Merck, Ref. 1077) just before use. For determinations of low concentrations of unlabelled betaxolol, these solvents were redistilled in glass. Pyridine (Merck, Ref. 7463) was also distilled. Bistrimethylsilyltrifluoro-

acetamide (BSTFA) and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), in 1 ml ampoules, were purchased from Pierce (Oud-Beijerland, Holland).

Biological samples

Blood plasma was obtained by N. Duval and P. Petruzzo, from dogs under pentobarbital anaesthesia. Some of the dogs had received betaxolol, during a study on the release of chronically administered betaxolol into the coronary circulation.[9] Control dogs had never received any β-blocker.

Samples of Krebs' bicarbonate buffer were obtained during a study of the electrically invoked release of betaxol from slices of the atrium of the rat.[10–11] Superfusates of the slices (about 3 mg/chamber) were collected in fractions of 2 ml. Each collection tube contained ($^{13}C_2$)betaxolol (500 pg; internal standard) and SL 76 020 (5 ng; 'carrier'). The procedure, carried out by M. Vidal, was as described earlier, except that some of the tissue slices were preloaded with ($^{2}H_7$)betaxolol instead of the tritiated analogue, which had a specific activity of 20 Ci mmol^{-1}. Usually, the slices are preloaded by incubating them with the radioactively labelled compound, whose release can be measured by scintillation counting. Our objective is to study the release phenomenon using tissues from animals which have been chronically pretreated with betaxolol, and for which the use of radioactivity cannot be envisaged.

Calibration curves

Stock solutions (100 μg ml^{-1}) of betaxolol and analogues were prepared in methanol, and stored at −20 °C. Just before use, dilutions of 100- to 1000-fold were made. In the work shown here, unlabelled betaxolol was determined using ($^{2}H_5$)betaxolol as internal standard, and calibration curves were prepared using either 5 or 50 ng of this, added to 1–2 ml aliquots of plasma from untreated dogs.

For the release experiments, ($^{2}H_7$)betaxolol was used as analyte, and calibration curves were prepared with 2 ml aliquots of Krebs' solution, using 200 or 500 pg ($^{13}C_2$)betaxolol as internal standard, and 5 ng SL 76 020 as 'carrier'.

Extraction of samples

The method described previously[1] was used, with minor changes. Tubes were kept in an ice-bath. Extractions were performed by vortexing for 10 s, and the phases were separated by centrifuging for a few seconds in a centrifugal evaporator (Savant Speedvac), which conveniently has a flashproof chamber.

The sample, treated with internal standard, carrier (where appropriate), and NaOH (2 M; 250 μl), was extracted with 3 ml purified ether. The ether was back-extracted with HCl (0.2 M; 1 ml), and then aspirated off. The aqueous phase was washed with 2 ml ether. Then, after addition of 250 μl of the NaOH, the betaxolol was extracted into 2 ml ether, and the upper layer transferred into small conical glass tubes fitted with B10/14 stoppers.

The solvent was evaporated using the centrifugal evaporator, taking care to eliminate the drop of water left behind by the ether. The samples were taken up in 10 or 20 μl BSTFA or MSTFA, either neat or diluted 1:1 with pyridine or acetonitrile. The tubes were either stored overnight in a desiccator at 4 °C, or else their tips were heated at 60 °C for 5 min. None of these variations in derivatization technique had any influence on the results. About half the betaxolol derivative decomposes within a week at 4 °C, but samples can be stored for a long time in a desiccator at −20 °C.

GC/MS

A Hewlett-Packard Model 5700 gas chromatograph was coupled to a VG Model 70-70F mass spectrometer. A capillary column switching system was used, essentially as described previously[12,13] for the determination of melatonin. The first column was 12 m × 0.32 mm bonded methyl silicone (SGE, Villeneuve St Georges, France; Ref. 12QC3/BP1-0.5), and the second column was 25 × 0.32 mm 5% phenylmethyl silicone (Ref. 25QC3/BP5-0.5).

Splitless injections (2–5 μl) were performed with the oven at 220 °C. Programming at 8 °C min^{-1} produced a broad peak on the first column at 4.25 min. Heart-cutting was performed between 3.5 and 5.0 min, the analyte being focused at the start of the second column by means of a liquid CO_2 trap. TMS-betaxolol eluted as a sharp peak at an overall retention time of (typically) 7.5 min. Chromatograms were recorded using a multichannel integrator (Trivector, Sandys, UK; Model 4000). Peak heights were measured from normalized recordings using a ruler, except when baseline noise was minimal.

The mass spectrometer was usually operated at a resolution of 3000 (10% valley), in EI mode (70 eV), with the ion source at 250–300 °C. For one experiment the collector slit was opened to give flat-topped peaks having a resolution of 700. The ions monitored (for 100 ms/cycle) for the different isotopomers of betaxolol are given in Table 1. Instrumental drift was corrected by scanning (for 50 ms/cycle) the molecular ion of 2-butanone (*m/z* 72.0575) or benzene (78.0469). In either case, ions from substances eluted from the column would occasionally interfere with the mass reference and drive the correction system out of range. One solution to this problem is to disenable the drift correction until about 1 min before elution of the analyte.

RESULTS

Mass spectra

Using the magnetic sector instrument, the *m/z* 72 fragment of TMS-betaxolol (Fig. 1) was even more prominent than in the previously published spectrum,[4] which had been obtained on a quadrupole instrument. An overloaded spectrum revealed the molecular ion,

together with M − H, at <0.01% relative intensity (Fig. 1, inset). The rearrangement ion at *m/z* 263 has already been explained.[4]

The labelled analogues of betaxolol gave spectra (not shown) having the expected isotopic shifts.

Determination of unlabelled betaxolol

Samples of plasma from an untreated dog were spiked with betaxolol, together with ($^2H_5$)betaxolol as internal standard. An excellent peak was obtained for 1 ng betaxolol (Fig. 2(a)). There was, however, a peak in the blank samples corresponding to 100 pg betaxolol (Fig. 2(b)), and the calibration curve (Fig. 3) did not pass through the origin. The peak in the blank samples was a chemical interference; the internal standard (which gives (*m/z* 74) was adequately labelled, and the degree of interference was about the same when this was replaced by ($^2H_7$)betaxolol (not shown). We were unable to improve the separation by changing the second gas chromatography column.

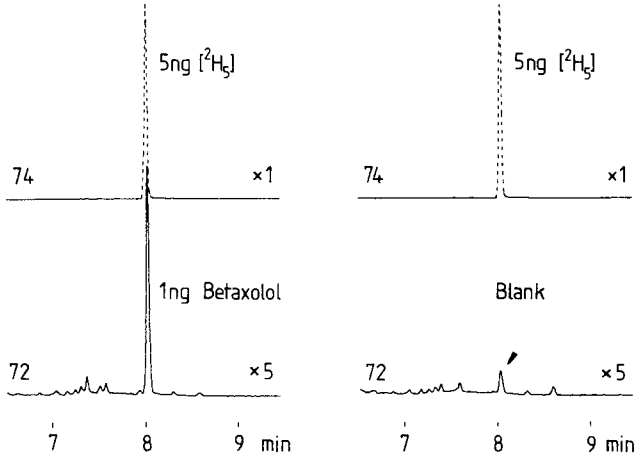


Figure 2. (a) Chromatogram for 1 ml dog plasma to which had been added 5 ng ($^2H_5$)betaxolol (internal standard) and 1 ng unlabelled betaxolol. (b) A 1 ml sample of the same plasma, containing only the internal standard.

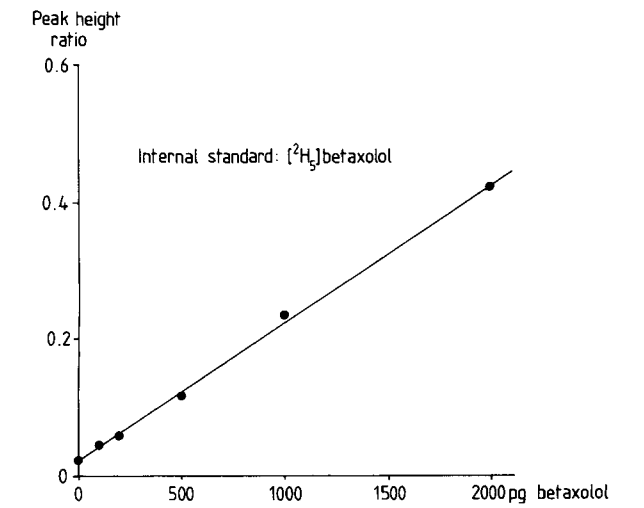


Figure 3. Calibration curve for unlabelled betaxolol added to dog plasma, using 5 ng ($^2H_5$)betaxolol as internal standard.

The interfering peak was more intense if samples, solvents or reagents had come into contact with disposable items such as polypropylene pipette tips or dispensers. Hydrocarbons are perhaps unlikely to be to blame, but an additive such as a light stabilizer of the 2,2,6,6-tetramethylpiperidine class[14] (or an impurity therein) is a possibility. Different batches of MSTFA and BSTFA contained varying amounts of the impurity, and this accounts for some of the interference appearing in Fig. 2.

Optimum mass spectral resolution for unlabelled betaxolol

When the resolution was degraded from 3000 to 700, the betaxolol peak became a minor feature of the chromatogram (Fig. 4); GC with low-resolution mass spectrometry gives poorer specificity than HPLC. By using the peak matcher, we found that most of the bleed and interfering peaks seen at 3000 resolution had an accurate mass very close to that of the betaxolol fragment. Thus there is little interest in further increasing the resolution, even where the sensitivity of the instrument permits.

Determination of labelled betaxolol

The traces obtained at 3000 resolution for the labelled forms of betaxolol have been consistently cleaner than those for the unlabelled compound. This was not the case at low resolution (Fig. 4). Figure 5 shows chromatograms obtained by spiking 2 ml aliquots of Krebs' solution with 0, 10 and 20 pg ($^2H_7$)betaxolol (analyte) together with 500 pg ($^{13}C_2$)betaxolol (internal standard). A 'carrier' (SL 76 020, 5 ng) was also added, although we have not systematically evaluated the necessity for this. With the present instrument, the limit of detection for the two isotopomers of betaxolol appears to be determined by the ion yield, as the baseline noise did not change appreciably when the accelerating voltage

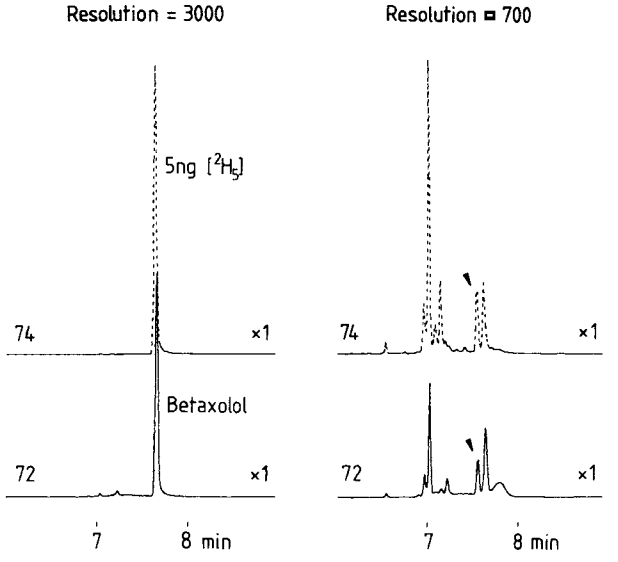


Figure 4. Chromatograms for a sample of plasma from a dog that had been treated with betaxolol: effect of varying the mass spectrometric resolution.

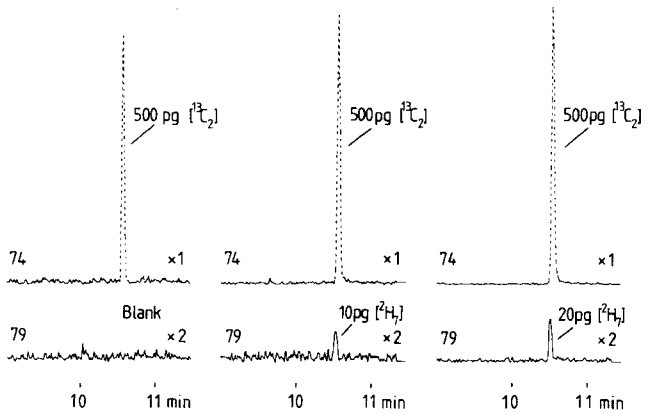


Figure 5. Chromatograms for 2 ml aliquots of Krebs' solution to which had been added the indicated amounts of ($^2H_7$)betaxolol (analyte) and ($^{13}C_2$)betaxolol (internal standard).

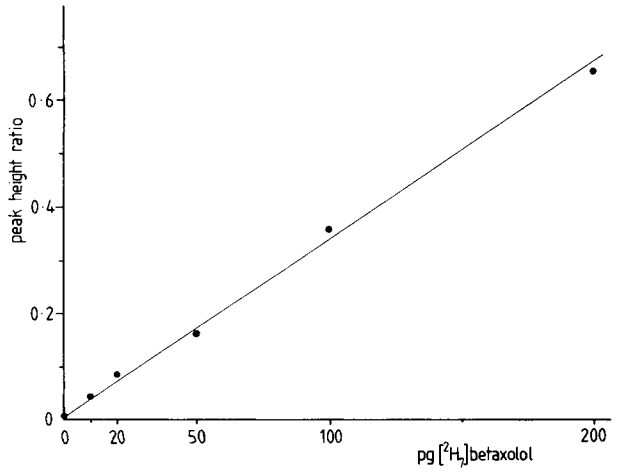


Figure 6. Calibration curve for ($^2H_7$)betaxolol added to 2 ml aliquots of Krebs' solution, using 200 pg ($^{13}C_2$)betaxolol as internal standard.

was switched off. There is a recognizable peak for 10 pg/tube (about 2 pg injected). A calibration curve (Fig. 6) for 0–200 pg per sample ($^2H_7$)betaxolol using 200 pg ($^{13}C_2$)betaxolol as internal standard was a straight line, with $r = 0.998$ ($N = 12$; points in duplicate). We have obtained similar calibration curves on three occasions.

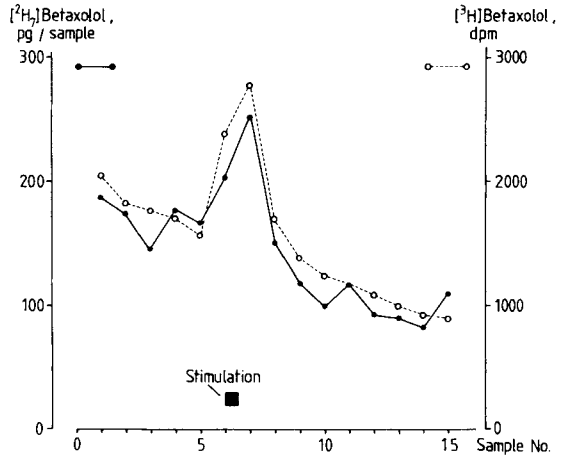


Figure 7. Demonstration of electrically evoked release of tritiated (dashed line) and deuterated analogues of betaxolol from superfused slices of rat atria.

Attempts to demonstrate electrically evoked release of unlabelled betaxolol from superfused tissue (see Methods) were unsuccessful, because the superfusate became contaminated by passage through plastic and rubber components in the apparatus (results not shown). With ($^2H_7$)betaxolol, however, results were comparable to those obtained by scintillation counting of the tritiated compound. Figure 7 shows the profiles obtained in an experiment where tissue slices that had been preloaded with the deuterated and tritiated compounds were placed in adjacent superfusion chambers (two chambers per isotope; the data were averaged). The rates of spontaneous release, as well as the amounts of betaxolol released by electrical stimulation, were comparable for the two isotopic forms, the only difference being that the data for deuterated betaxolol show rather more scatter.

DISCUSSION

It is a good general rule in quantitative analysis by GC/MS that the derivative and ionization method chosen should yield one or more prominent characteristic ions in the high-mass region of the spectrum. Aliphatic amines which undergo α-cleavage are among the rare exceptions. The most sensitive assays available for several '*tert*-butylamino' β-blockers involve SIM of the *m/z* 86 fragment.[5–7] Acetylcholine is determined with picomole sensitivity by GC/MS of the product of demethylation, *O*-acetyl-*N*,*N*-dimethylaminoethanol, the homologous fragment *m/z* 58 being monitored.[15] This method was described for use with quadrupole instruments, but we have found that the baseline noise can be reduced considerably by operating at medium resolution (unpublished observation). Cohen and Jemal[16] have determined carboxylic acids by converting them to (diethylamino)ethyl esters, and monitoring the corresponding intense α-cleavage fragment. A major advantage of this approach is that it enables the use of EI ionization, which minimizes contamination of the mass spectrometer, and is the only technique available with inexpensive 'desk-top' instruments.

Particular care is needed in evaluating the specificity of methods involving SIM of low-mass fragments. This has been the subject of a polemic,[17,18] but in practice checking for interferences often presents little difficulty in pharmaceutical research. With low resolution mass spectrometry, betaxolol (monitored at *m/z* 72) was rather more prone to interference in the low nanogram per sample range than drugs which are monitored at *m/z* 86,[5–7] and since there already exists a sensitive HPLC method for betaxolol[3] we have not pursued this approach. However, for small fragments, a resolution of only a few thousand is sufficient to discriminate between the analyte ion and the majority of isobaric ions which would interfere at low resolution. Interference by metastable ions may be relatively more likely at low mass; we could not use perfluorokerosine as mass reference, because it gives an abundance of weak metastable peaks below mass 100, but otherwise we have experienced no difficulties.

Ultimately, the specificity may be limited by the presence of interfering ions having the same elemental composition as that of the analyte, as we found for unlabelled betaxolol. In our case, the problem could be dealt with, as it was due to impurities in the solvents and reagents. We have not yet attempted to remove all sources of contamination, for example by purifying a silylating reagent. However, the method is already of value in studies for which the sensitivity of the HPLC method is marginal, and where contamination of the samples can be controlled.

There is scope for improving the chromatographic separation, as we have not yet been able to exploit the full separating power of the column-switching system. The chromatographic conditions used for the first column could be modified to give a sharper peak, but this would prolong the analysis time, particularly for splitless injections, and the timing of the heart-cut would then become unduly critical. A first column of 0.53 mm i.d. instead of 0.32 mm gives relatively sharp peaks and short retention times, while allowing the analysis to be performed isothermally. However, some further development will be required in order to achieve retention times sufficiently stable throughout the day for precise heart-cutting (unpublished work). The separation could also be improved by using columns of widely different selectivities, but the range of polar columns sufficiently thermostable for a general-purpose installation is unfortunately limited.

In contrast to unlabelled betaxolol, the analogues labelled with stable isotopes present no analytical problems at all for concentrations down to a few picograms per sample, and comparable results should be obtainable for several other drugs which give the same fragment. The deuterated and ^{13}C-labelled analogues could be used interchangeably for the *in vitro* experiment illustrated here (Fig. 7), but the ^{13}C analogue may be the more appropriate analyte where isotope effects must be taken into account. Labelling with two atoms per molecule of ^{13}C is not unduly expensive, since the isotope is introduced at the final step in the synthesis.

Acknowledgements

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