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Determination of (−)-bunolol and its metabolite, dihydro-(−)-bunolol, in human aqueous humour by gas chromatography–negative ion chemical ionisation mass spectrometry

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ABSTRACT

(−)-Bunolol (LB) was applied to the human eye in a commercially available eye drop formulation. LB and its metabolite, dihydro-(−)-bunolol (DHLB) were identified and quantified in human aqueous humour. The compounds were analysed as their trimethylsilyl-pentafluorobenzamide derivatives using gas chromatography–negative ion chemical ionisation mass spectrometry. In the case of DHLB the corresponding $^2H_3$-labelled isotopomers were used as internal standards and LB was quantified against its methoxime derivative. Calibration curves for LB and DHLB against internal standards were linear with correlation coefficients 0.994 and 0.996, respectively. Replicate analyses of a pooled sample of aqueous humour containing LB and DHLB gave standard errors of the mean of ± 9.8 and ± 2.4% for the concentrations of LB and DHLB, respectively. The practical limit of detection of the method was *ca.* 30 pg for LB and *ca.* 100 pg for DHLB. The derivatization procedure was also satisfactory for the analysis of a number of other β-blockers which are used in ophthalmological practice.

INTRODUCTION

β-Blockers, formulated as eye drops, are widely used in ophthalmological practice to control intraocular pressure in cases of glaucoma [1,2]. There are no data concerning the effectiveness of different β-blocker preparations in penetrating the human eye or on the time course for the elimination of the drug from the aqueous humour. It is likely that β-blockers act by binding to receptors in the ciliary body thus reducing the rate of production of aqueous humour [3,4]. Optimisation of dosage of β-blocker may be important; particularly in the elderly, where administration of excess of the preparation, usually applied twice daily over long periods, via the tear duct and nasal passage may have unwanted side-effects.

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We have employed gas chromatography–negative ion chemical ionisation mass spectrometry (GC–NICIMS) in previous studies on the absorption of steroids into the human eye [5–7] and have found that the sensitivity and specificity of the technique were vital for the accurate and precise analysis of the small volumes of sample involved. In earlier work we developed an aqueous phase derivatisation technique for the analysis of biogenic amines [8–10] and found that the most satisfactory results were obtained when the amine was acylated with ditrifluoromethylbenzoyl chloride. In this case, when the derivative was analysed by GC–NICIMS, most of ion current was carried by the molecular ion of the derivative [8], which gave a high degree of sensitivity and specificity. We now report an extension of our earlier work on aqueous phase derivatisation to the analysis of β-blockers and the application of the technique to the determination of (−) bunolol (LB, Fig. 1) and its metabolite, dihydro-(−)-bunolol (DHLB, Fig. 1), in human aqueous humour.

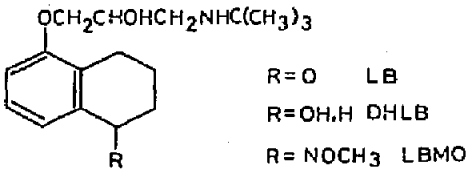

Fig. 1. Structures of (−)-bunolol, dihydro-(−)-bunolol and methoxime of (−)-bunolol.

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: LB · HCl and DHLB · hemifumarate (a gift from Allergan UK); betaxolol, carteolol, metipranolol and timolol were extracted from commercially available preparations of eye drops; bistrimethylsilylacetamide (BSA) (Fluka, Glossop, UK); ^2HCl, ^2H_2O, CH_3COO^2H, $C_2H_5O^2H$, NaB^2H_4 (Aldrich, Gillingham, UK); pentafluorobenzoyl chloride (PFBCl) (Fluorochem, Glossop, UK); all solvents were HPLC grade (Rathburn, Walkerburn, UK).

Internal standards

[$^2H_3$]DHLB was prepared as follows: LB · HCl (200 mg) was dissolved in ^2HCl (5 ml, 20% in ^2H_2O) and heated (60°C, 2 h) in a sealed tube. The solution was made alkaline by addition of sodium methoxide (1.6 g): the resultant precipitate was extracted into diethyl ether (2 × 5 ml) and dried (anhydrous sodium sulphate). The solvent was removed, the unpurified product was dissolved in $C_2H_5O^2H$ (5 ml), and NaB^2H_4 (200 mg) was added. The suspension was refluxed (18 h) and cooled, and CH_3COO^2H (1.0 ml) was added; then the solution was diluted to 20 ml with water and made alkaline by the addition of 5 *M* sodium

hydroxide. The solution was extracted with diethyl ether (2 × 30 ml), and the combined organic layers were dried (anhydrous sodium sulphate). The solvent was removed affording crude product (83 mg) as a gum. This was used as an internal standard without further purification. Analysis by GC–NICIMS indicated that the isotope composition [based on the molecular ion of the pentafluorobenzamide-di(trimethylsilyl) (PFB-diTMS) derivative] was as follows: $^2H_3$, 96.3%; $^2H_2$, 1.8%; $^2H_1$, 1.8%; $^2H_0$, 0.1%.

The methoxime of LB (Fig. 1) was prepared as follows: LB · HCl (50 mg) was heated with methoxylamine · HCl (50 mg in 1 ml of pyridine) for 1 h at 80°C. Most of the pyridine was removed by evaporation under a stream of nitrogen at 80°C, sodium hydroxide (2 ml, 2 *M*) was added to the residue and the resultant emulsion was extracted with diethyl ether (2 × 2 ml) and dried (anhydrous sodium sulphate). The solvent and excess methoxylamine were removed by evaporation under a stream of nitrogen at 60°C. This internal standard also was used without further purification.

Samples

Samples of aqueous humour were obtained from patients undergoing routine cataract surgery and informed consent was obtained in all cases. LB · HCl (0.5%, 50 μl, Betagan, Allergen UK) was introduced into the eye at various time intervals before surgery. Collection of the aqueous humour has been described in full previously [6,7].

Derivatisation procedure

The sample of aqueous humour (40–80 μl) was diluted to 1 ml with 0.2 *M* potassium hydroxide, and [$^2H_3$]DHLB and LB methoxime (20 μl of a 2 ng/μl solution of each in acetonitrile) were added. The solution was extracted with diethyl ether (2 ml) and the extract dried by passage through anhydrous sodium sulphate in a Pasteur pipette. The ether layer was removed under a stream of nitrogen, the residue was then dissolved in methanol (100 μl) and 0.2 *M* potassium hydroxide (1.4 ml) was added. PFBCl (5 μl) was added, and the mixture was shaken for 5 min (until it was clear). The solution was then extracted with diethyl ether (3 ml) and the extract dried by passage through anhydrous sodium sulphate in a Pasteur pipette. The ether layer was evaporated under a stream of nitrogen, the residue was dissolved in BSA (30 μl) and the solution heated for 30 min at 60°C. The volume of the reaction mixture was reduced to *ca.* 5 μl with a stream of nitrogen and then it was diluted with ethyl acetate (80 μl). An aliquot (4 μl) of the final solution was injected into the GC–MS system.

Quantitation

In order to establish linearity for the method, calibration curves were constructed by derivatising 40 ng of internal standards with varying amounts (1–100 ng) of the corresponding unlabelled compound as described above. The cali-

bration curve for DHLB was constructed by plotting the sum of the peak areas of both deuteriated diastereomers against the peak area of the undeuteriated standard. In order to correct for instrumental variation on a day-to-day basis quantitation was determined using a 1:1 standard mixture with each batch of samples, and a reagent blank was run with each batch of samples. The reproducibility of the method was checked on three replicate analyses from a pooled sample of aqueous humour collected from subjects who had had LB instilled into their eyes.

Instrumentation

GC–MS in the NICI mode was carried out using a Hewlett-Packard 5988A GC–MS instrument with an RTE6V/M data system. Methane was used as the reagent gas with a source pressure of *ca.* 263 Pa and a temperature of 140°C. Linear scans were carried out over the range 100–800 a.m.u. The Hewlett-Packard 5890 gas chromatograph was fitted with a cross-linked methyl silicone column (12 m × 0.25 mm I.D., film thickness 0.25 μm, SGE BP-1, Burke Electronics, Glasgow, UK). Temperature programme conditions were as follows: 140°C for 1 min, then 30°C min^{-1} to 280°C (10 min). Injector temperature was 250°C and interface temperature 280°C. Injections were made in the splitless mode.

RESULTS AND DISCUSSION

The derivatisation procedure may be explained as follows: the initial extraction of the amines under basic conditions is required to remove them from traces of protein which interfere with the aqueous phase acylation by coagulating with the PFBCl thus bringing it out of solution. The extracted amines are initially redissolved in a small amount of methanol since they might otherwise stick to the wall of the sample tube on addition of the second quantity of 0.2 *M* potassium hydroxide. It is important to allow all the PFBCl to hydrolyse and be converted to its potassium salt before extraction since extraction of the reagent interferes with the final analysis. Use of ethyl acetate rather than diethyl ether in the second extraction step caused potassium pentafluorobenzoate to extract as an ion pair and this occluded the derivatised amines in the extract preventing their derivatisation with BSA. Finally, we have found that a small amount of BSA in the sample improves the sensitivity of the method especially where a column has been in use for some time.

The NICI mass spectrum of the PFB-TMS derivative of LB (Fig. 2) is similar to the type of mass spectrum shown by corresponding derivatives of biogenic amines in our earlier studies [8–10]: a particular feature being that the ion current is carried largely by the molecular ion of the derivative. The mass spectrum of the PFB-TMS derivative of the methoxime of LB is similar (with the difference between the molecular ions being 29 a.m.u.).

Figs. 3 and 4 show the mass spectra of the PFB-diTMS derivatives of DHLB and [$^2H_3$]DHLB, and again it is apparent that most of the ion current is carried

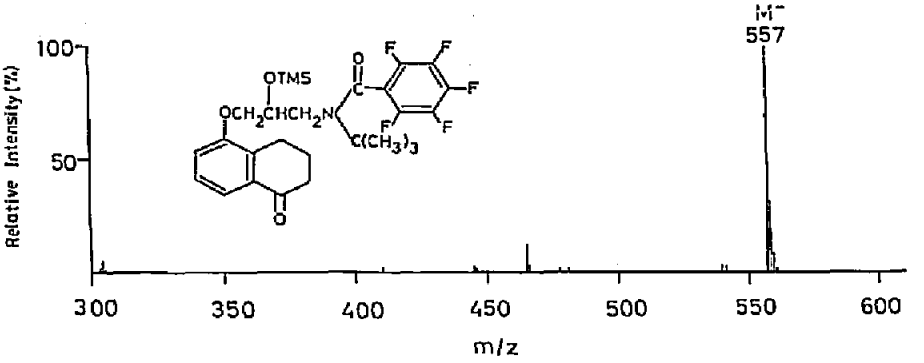

Fig. 2. NICI mass spectrum of (−)-bunolol as the PFB-TMS derivative.

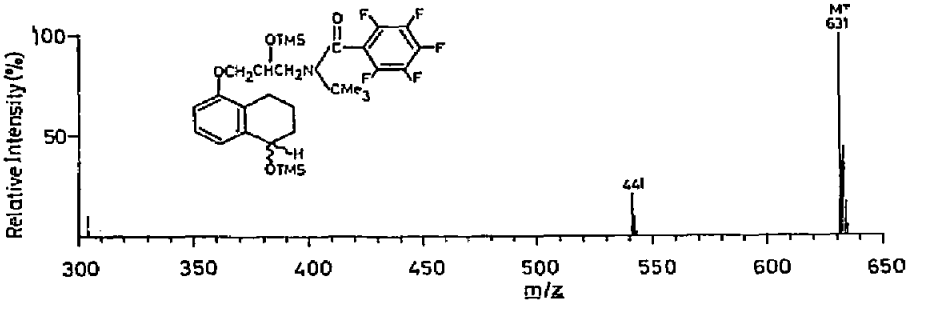

Fig. 3. NICI mass spectrum of dihydro-(−)-bunolol as the PFB-diTMS derivative.

by the molecular ion. The response of the instrument under NICI conditions to the PFB-TMS derivative of LB was two to three times that of the PFB-diTMS derivative of DHLB; this is probably due to the enhanced electron-capturing effect conferred by the conjugated keto group in the former. It was found that the methoxime of LB had similar electron-capturing properties to those of LB and consequently was more suitable as an internal standard for LB than [$^2H_3$]DHLB. Calibration curves for LB and DHLB were linear over the range 1–100 ng with

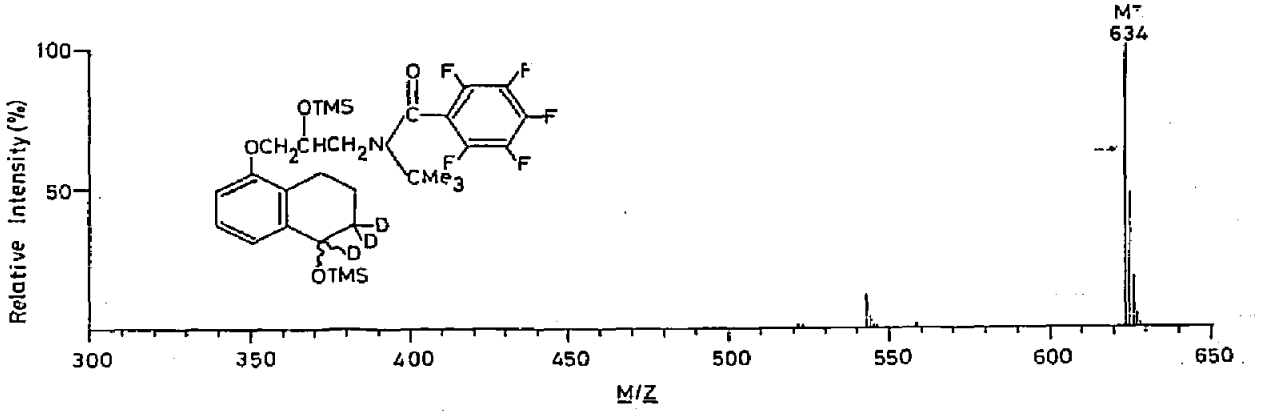

Fig. 4. NICI mass spectrum of [$^2H_3$]dihydro-(−)-bunolol as the PFB-diTMS derivative.

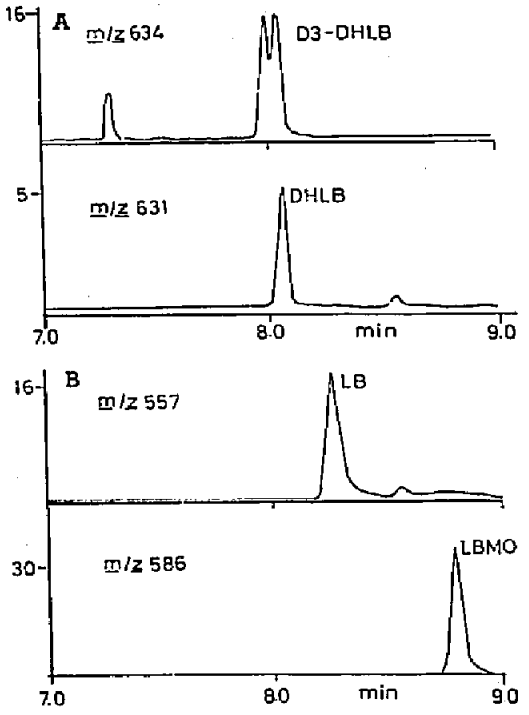

Fig. 5. SIM trace showing (A) dihydro-(−)-bunolol (67.4 ng ml^{-1}) and (B) (−)-bunolol (112.6 ng ml^{-}) extracted from human aqueous humour 24 min after topical application of Betagan (50 μl) in comparison to 40 ng of [$^{2}H_{3}$]dihydro-(−)-bunolol and 40 ng of (−)-bunolol methoxime added to the aqueous humour before extraction and derivatisation.

correlation coefficients of 0.994 and 0.996, respectively. The reproducibility of the analytical method was checked on three replicate analyses from a pooled sample of aqueous humour containing 125.9 ng ml^{-1} DHLB nd 35.5 ng ml^{-1} LB. The standard errors of the mean (S.E.M.) were ± 2.4% for DHLB and ± 9.8% for LB, the lower precision for LB reflecting the use of an analogue as an internal standard. Fig. 5 shows selected-ion monitoring (SIM) traces of a derivatised extract from human aqueous humour taken 24 min after instillation of the drop

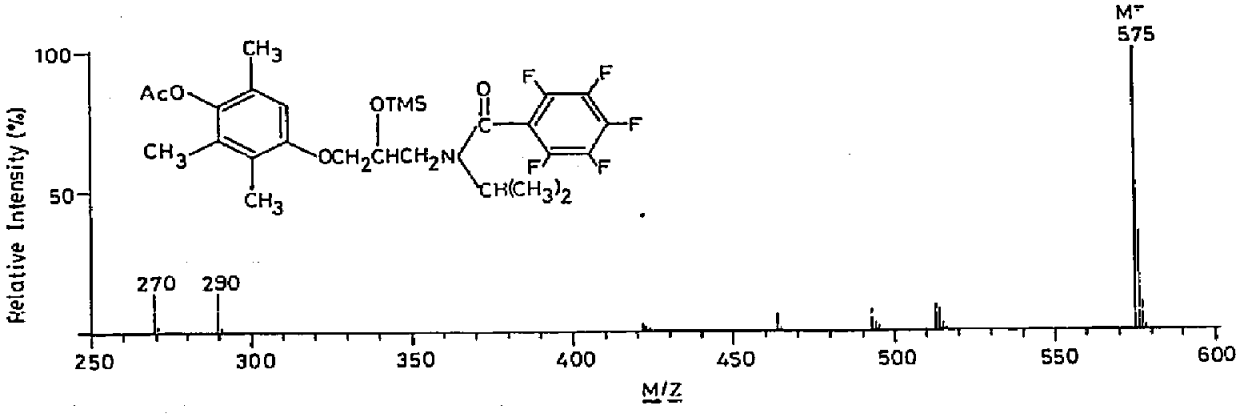

Fig. 6. NICI mass spectrum of metipranolol as the PFB-TMS derivative.

TABLE I

NICIMS DATA OF PFB-TMS DERIVATIVES OF β-BLOCKERS USED IN THE TREATMENT OF GLAUCOMA

β-Blocker	*I* value	Base peak (*m/z*)	Other ions in mass spectrum
(−)-Bunolol	2961	557[a]	465(13), 284(13), 161(11)
(−)-DHLB	2942, 2948	631[a]	541(21), 304(10), 284(15)
(−)-Bunolol methoxime	3051	586[a]	494(3), 284(9)
Metipranolol	2844	575[a]	513(9), 290(15), 270(14)
Timolol	2853	582[a]	284(3)
Betaxolol	2968	573[a]	290(8), 270(8)
Carteolol	3159	538	558[a](38), 466(28)

[a] Molecular ion.

and containing 67.4 ng ml^{-1} DHLB and 112.6 ng ml^{-1} LB. Trace B shows LB (*m/z* 557) in comparison with LB methoxime (*m/z* 586). Trace A shows DHLB (*m/z* 631) in comparison with [$^2H_3$]DHLB (*m/z* 634), the single peak for DHLB arising from a single diastereomer and indicating the stereospecific nature of the reductase involved in the biological reduction. In contrast the non-stereospecific reduction with sodium borodeuteride used in making the internal standard resulted in two diastereomers which have different retention times, as their PFB-TMS derivatives, on GC. Quantitation of DHLB against the total area for both deuteriated diastereomers proved satisfactory because the recovery of the two diastereomers was always identical. The use of the diastereomeric mixture was also useful because it helped to highlight the stereospecificity of the biological reduction, *i.e.* confirm in each case that resolution between the diastereomers was maintained.

It has been established previously that LB is rapidly metabolised during its passage through the cornea in rabbit eye [11]. A full report concerning the penetration and metabolism of the drug in the human eye will be made in the ophthalmological literature by us when the current study is complete. In brief, the data obtained so far indicate that LB attains maximum concentrations of *ca.* 500 ng ml^{-1} 20–40 min after its topical application to the eye, whilst DHLB attains maximum concentrations of *ca.* 400 ng ml^{-1} 90–120 min after application of the drop.

The analytical procedure was also applied to a number of other β-blockers used in ophthalmological practice. Fig. 6 shows the NICI mass spectrum for metipranolol, and again it may be seen that the ion current is carried mainly by the molecular ion of the derivative. Table I summarises the NICIMS data for the PFB-TMS derivatives of the β-blockers currently used in controlling intraocular pressure. The molecular ion is a major fragment in all cases. It is hoped that the

results of our further studies may lead to the design of improved dosage regimens for administration of these drops.

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