Journal of Chromatography, 518 (1990) 215–220
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 632

Note

Identification using solid phase extraction and gas chromatography–mass spectrometry of timolol in equine urine after intravenous administration

A. M. DUFFIELD*, S. WISE, J. KELEDJIAN and C. J. SUANN
Australian Jockey Club Laboratory, P.O. Box 3, Randwick, N.S.W. 2031 (Australia)
(Received May 15th, 1990)

Drugs belonging to the β-blocker group have the potential to curtail cardiac output in the horse and their possible use as "go-slow" drugs is a continuous concern of the racing industry [1]. Timolol, *S*-1-*tert*-butylamino-3[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol is a β-blocker used in the treatment of hypertension and angina [2] and to reduce intra-ocular pressure experienced in glaucoma [3].

Methods for the detection and identification of timolol (available as Blocadren) has been described using capillary gas chromatography (GC)–flame ionization detection (FID) and GC–mass spectrometry (MS) [4]. Solid-phase extraction has proved useful in the screening of many other β-blocker drugs [5,6]. We now describe a solid-phase extraction procedure for the isolation of timolol from equine urine and its identification by GC–MS. This methodology was responsible for the identification of timolol in separate post race urine samples taken at Sydney racecourses from three beaten favourites whose individual performances were far below expectation. A procedure is also described for the routine post race screening of timolol in equine urine using a liquid–liquid extraction procedure.

EXPERIMENTAL

Instrumentation

A Finnigan-MAT Incos 50 GC–MS system was utilised in this study. Electron impact (EI) ionisation operating with 70-eV electrons and a trap current of 750 μA was used with the ion source temperature maintained at 190°C and the GC transfer line at 270°C. The GC column (J & W Scientific, Davis, CA, U.S.A., DB-5, 15 m × 0.3 mm I.D.), operated with helium as carrier gas (flow is 1 ml min^{-1}). The initial GC oven temperature of 100°C was programmed to 300°C at 30°C min^{-1} commencing 2 min after sample injection. The mass spectrometer was repetitively scanned from *m/z* 50 to *m/z* 450 in 0.6 s.

0021-9673/90/$03.50 © 1990 Elsevier Science Publishers B.V.

Chemicals

Timolol was supplied by Merck Sharp and Dohme Australia. Solvents were of nanograde quality and were purchased from Mallinckrodt, Meadowbank, Australia. Bond Elut Certify columns were produced by Analytichem and purchased from F.S.E., Homebush, Australia.

Drug administration

Timolol (30 mg) in sterile water (30 ml) was administered intravenously (i.v.) to a healthy Thoroughbred mare and urine samples collected after 2, 4, 6, 8 and 12 h. These samples were stored frozen until analysed.

Solid-phase extraction procedure

Equine urine (50 ml, 2 h administration) was adjusted to pH 6.1 with hydrochloric acid and filtered through a Whatman No. 1 filter paper. Four Bond Elut Certify cartridges were each primed with methanol (2 ml) followed by deionised water (2 ml). Filtered urine (5 ml) was passed through each of the four cartridges which were successively rinsed with water (2 ml), sodium acetate buffer (0.1 *M*, pH 4, 1 ml) and methanol (2 ml). Freshly prepared dichloromethane–isopropanol–conc. NH_4OH (8:2:0.2; 2 ml) was used to elute each cartridge. The combined eluents (8 ml) were

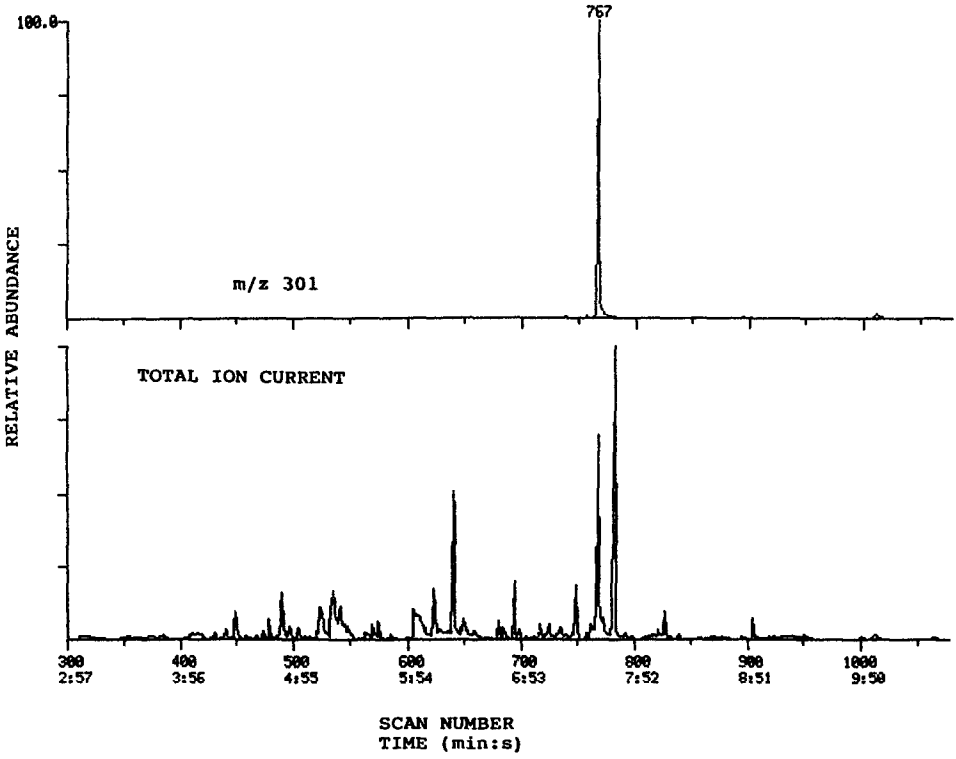

Fig. 1. Total ion current (TIC) trace of an equine urine (2 h administration) extract (lower) and profile for *m/z* 301 indicating the elution of timolol at scan number 767.

concentrated at 70°C under nitrogen gas. The residue was dissolved in methanol (20 μl) and aliquots (2 μl) used for GC–MS analysis.

Screening procedure for timolol in urine

Post-race urines were composited (5 × 2 ml), their pH adjusted to 5.0 and hydrolysed overnight at 37°C with the mixed β-glucuronidase-arylsulphatase enzyme from *Helix pomatia* (Boehringer Mannheim, F.R.G.). The urine composite was adjusted to pH 2–3 with hydrochloric acid and two drops of 10% sodium metabisulphite added and the sample extracted on a rotorack with ethyl acetate (5 ml) for 5 min. After centrifugation the organic layer is aspirated and discarded.

The composite urine sample was then adjusted to pH 9.0–9.5 with concentrated NH_4OH and extracted with a mixture of dichloromethane–isopropanol (4:1; 3 ml). After centrifugation the aqueous layer was aspirated and discarded. Acetic anhydride (one drop) was added and the solvent evaporated at 70°C under nitrogen. The residue was then reacted with acetic anhydride (one drop) in pyridine (two drops) at 80°C for 20 min. Sulphuric acid (0.1 *M*, 1 ml) and dichloromethane (2 ml) were added and the tube thoroughly vortexed. The aqueous layer was transferred to a Kimble Tube, $NaHCO_3$ (50 mg) added, followed by 10% NH_4OH (one drop). Dichloromethane (5 ml) was added, the two phases thoroughly vortexed, and after separation, the aque-

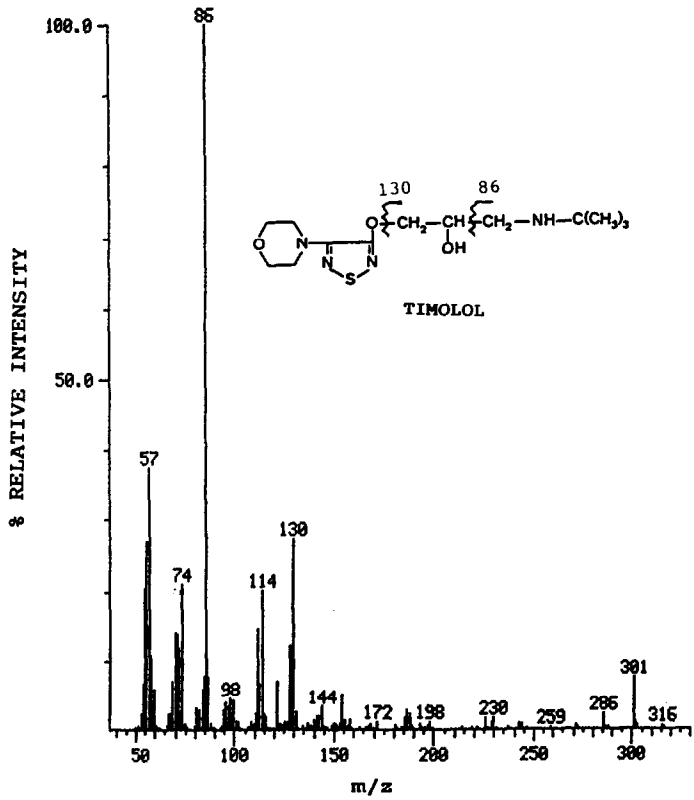

Fig. 2. EI mass spectrum of timolol corresponding to scan number 767 in Fig. 1.

ous phase discarded. The organic phase was dried (Na_2SO_4) and, following the addition of acetic anhydride (one drop), blown to dryness at 70°C under nitrogen.

The residue was reconstituted in isopropanol (30 μl) and an aliquot (2 μl) injected into a Hewlett-Packard MSD Model 5970 GC–MS system equipped with an Autosampler (Ryde, Australia). The GC column was an Ultra 1 plus methyl silicone (Hewlett-Packard; 12 m, 0.23 mm I.D., coating thickness 0.33 μm). GC analysis was accomplished using an initial oven temperature of 130°C, which was maintained for 1 min after sample injection, programmed at 30°C min^{-1} to 280°C and held at this temperature for 1 min. Methyl stearate served as an internal standard for retention time (4 min 58 s) and timolol acetate eluted at 5 min 40 s.

This procedure could readily detect timolol acetate after processing urine from the 2- and 4-h i.v. drug administration samples which were used as positive controls for each batch analysis (20 composited samples representing 100 urines).

RESULTS AND DISCUSSION

Fig. 1 records the GC–MS response obtained from the extract of a urine sample collected 2 h after i.v. administration of timolol (30 mg). The drug eluted from the GC–MS system (Incos 50) as a sharp peak and its mass spectrum is recorded in Fig. 2.

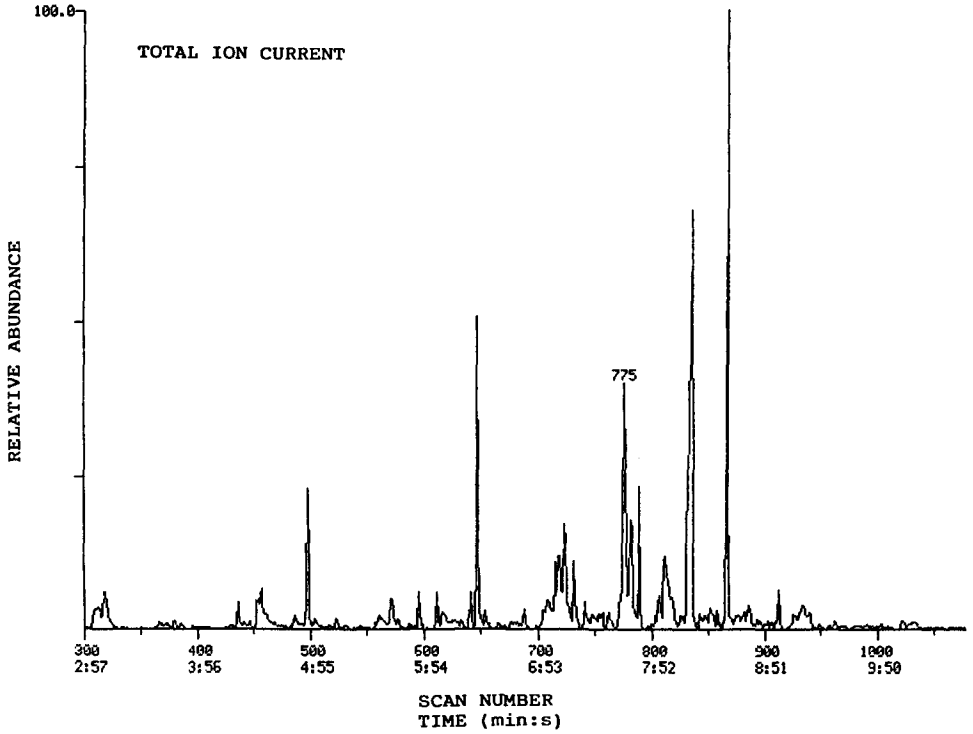

Fig. 3. TIC trace of an acetylated extract of equine urine after i.v. timolol administration. Timolol acetate elutes at scan number 775.

An identical GC retention time (7 min 38 s, scan 767) and mass spectrum was obtained after the analysis of a standard timolol solution.

When the urine extract from solid-phase extraction was acetylated (acetic anhydride in pyridine at room temperature overnight) timolol acetate could be identified (TIC reproduced as Fig. 3) and its mass spectrum is reproduced in Fig. 4. The acetate had a retention time of 7 min 43 s (scan 775 in Fig. 3) (Incos 50 GC–MS system) and its mass spectrum [7] was identical with an authentic standard prepared from timolol.

Following i.v. administration of timolol (30 mg) this methodology could detect the drug in equine urine up to 4 h. If timolol (50 mg) was administered orally (stomach tube) no free drug was detected in urine after either 2 or 4 h. Hydrolysis of urine after i.v. administration with the mixed β-glucuronidase-arylsulphatase enzyme from *Helix pomatia* resulted in approximately a two-fold increase in the amount of timolol detected as compared to non-hydrolysed urine.

Subsequent to the identification of timolol in the urine of three horses, who competed and failed badly at Sydney racecourses as short priced favourites, this laboratory instituted a GC–MS screen for timolol derivatised as its acetate. This

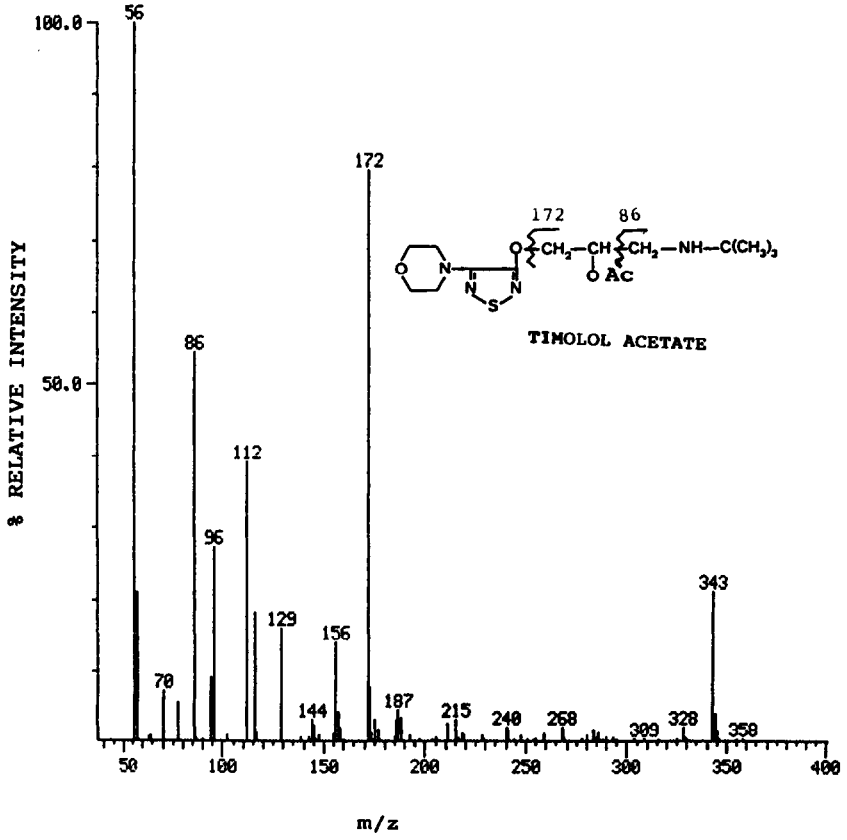

Fig. 4. EI mass spectrum of timolol acetate corresponding to scan number 775 in Fig. 3.

method used selected ion monitoring (SIM) of m/z 172 in the mass spectrum of timolol acetate (Fig. 4) isolated from five composite urine samples by liquid–liquid extraction with dichloromethane–isopropanol (4:1). Solvent extraction was used rather than solid phase extraction for the screening of routine samples because of the increased sample throughput and cost savings.

The base peak in the mass spectrum of timolol (Fig. 2), and a prominent ion in its acetate derivative (Fig. 4), occurs at m/z 86 and its origin is consistent with α-cleavage to the aliphatic nitrogen atom in both compounds. The ion of mass 130 in the mass spectrum of timolol is located at m/z 172 in the mass spectrum of its acetate derivative (Fig. 4) and would result from the indicated bond fissions. Low intensity molecular ions were observed in the mass spectra of timolol and its acetate at m/z 316 and m/z 358, respectively. A more abundant ion species occurs from the loss of a methyl radical from each molecular ion (see m/z 301 and m/z 358 in Figs. 2 and 4, respectively).

ACKNOWLEDGEMENTS

Timolol was a generous gift from Merck Sharp and Dohme Australia. We thank the Racecourse Development Committee of New South Wales for the purchase of the GC–MS systems and for laboratory facilities.

REFERENCES

1 M. C. Dumasia and E. G. Houghton, *Biomed. Environ. Mass. Spectrom.*, 18 (1989) 1030.
2 N. Weiner, in A. Goodman Gilman, L. S. Goodman, T. W. Rall and F. Murad (Editors), *The Pharmacological Basis of Therapeutics*, 7th Ed., MacMillan, New York, 1985, p. 200.
3 H. Bundgaard, A. Burr and V. H. L. Lee, *Acta Pharm. Suec.*, 25 (1988) 293.
4 R. D. Rofi and G. E. Aldoma, *J. High Resolut. Chromatogr.*, 12 (1989) 428.
5 P. M. Harrison, A. M. Tonkin and A. J. MacLean, *J. Chromatogr.*, 339, (1985) 429.
6 M. S. Leloux, E. G. de Jong and R. A. A. Maes, *J. Chromatogr.*, 488 (1989) 357.
7 K. Pfleger, H. Maurer and A. Weber, *Mass Spectra and GC Data of Drugs, Poisons and their Metabolites, Part II, Mass Spectra and Indexes*, VCH, Deerfield Beach, FL, 1985, p. 614.