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In vivo biotransformation of metoprolol in the horse and on-column esterification of the aminocarboxylic acid metabolite by alcohols during solid phase extraction using mixed mode columns

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Abstract

The in vivo biotransformation of metoprolol tartrate in the thoroughbred racehorse was studied after administration of a single oral dose. Metoprolol and its basic and bifunctional phase I metabolites were isolated from urine and plasma using mixed mode solid phase extraction (SPE) cartridges. The isolates were derivatised as trimethylsilyl ethers and analysed by capillary column gas chromatography—positive ion electron ionisation and ammonia chemical ionisation mass spectrometry. Metabolism was primarily confined to the oxidative transformations of the *p*-(2-methoxy)ethyl substituent. Metoprolol and five phase I metabolites were detected in horse urine. In common with man, rat and dog, the zwitterionic compound (±)-4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetic acid (H117/04), was the principle metabolite in the horse. This compound was readily isolated from both plasma and urine samples by SPE and, in addition, an unusual on-column esterification of the carboxylic acid moiety by alcohols was observed. Metoprolol and the major aliphatic acid metabolite were detected for about 10 and 40 h, respectively in unhydrolysed urine. After enzymatic hydrolysis, the detection period increased to 15 and 60 h, respectively indicating some phase II metabolism of metoprolol and its metabolites in the horse.

Keywords: Metoprolol; Horse; Biotransformation; Aminocarboxylic acid metabolite; Fischer esterification; GC/MS

1. Introduction

Metoprolol {(±)-1-isopropylamino-3-[4-(2-(methoxyethyl)phenoxy]-propan-2-ol} is a β_1-cardioselective adrenoceptor antagonist used in the treatment of cardiovascular disorders [1]. Because of relatively high first-pass metabolism, approximately 50–60% of an oral dose reaches systemic circulation [2] and less than 5% is excreted unchanged in urine in 24 h [3]. Metabolism of metoprolol has previously been reported in man, rat and dog [4–6]. It is extensively metabolised by oxidative pathways genetically linked to debrisoquine and bufuralol hydroxylation [7,8]. Phase I biotransformations include oxidative deamination, benzylic (aliphatic) hydroxylation and *O*-dealkylation with subsequent oxidation of the alcohol intermediates to aliphatic acids. Metoprolol and its metabolites are excreted in urine mainly in the unconjugated form [4]. Difficulties have been encountered in the extraction of the major bifunctional metabolite H117/04 from biofluids [3].

Methods for the detection of metoprolol and its metabolites in biological matrices are based upon solvent or SPE and detection by gas chromatography (GC) with electron capture detection of perfluoroacyl derivatives [9] and nitrogen sensitive detection of oxazolidinone derivatives [10]. Detection of metoprolol and its metabolites by capillary GC–mass spectrometry (GC/MS) as oxazolidinone-trimethylsilyl [11] perfluoroacyl [12] and mixed TFA–TMS [13] has been described. Metoprolol and its metabolites have also been analysed by high performance liquid chromatog-

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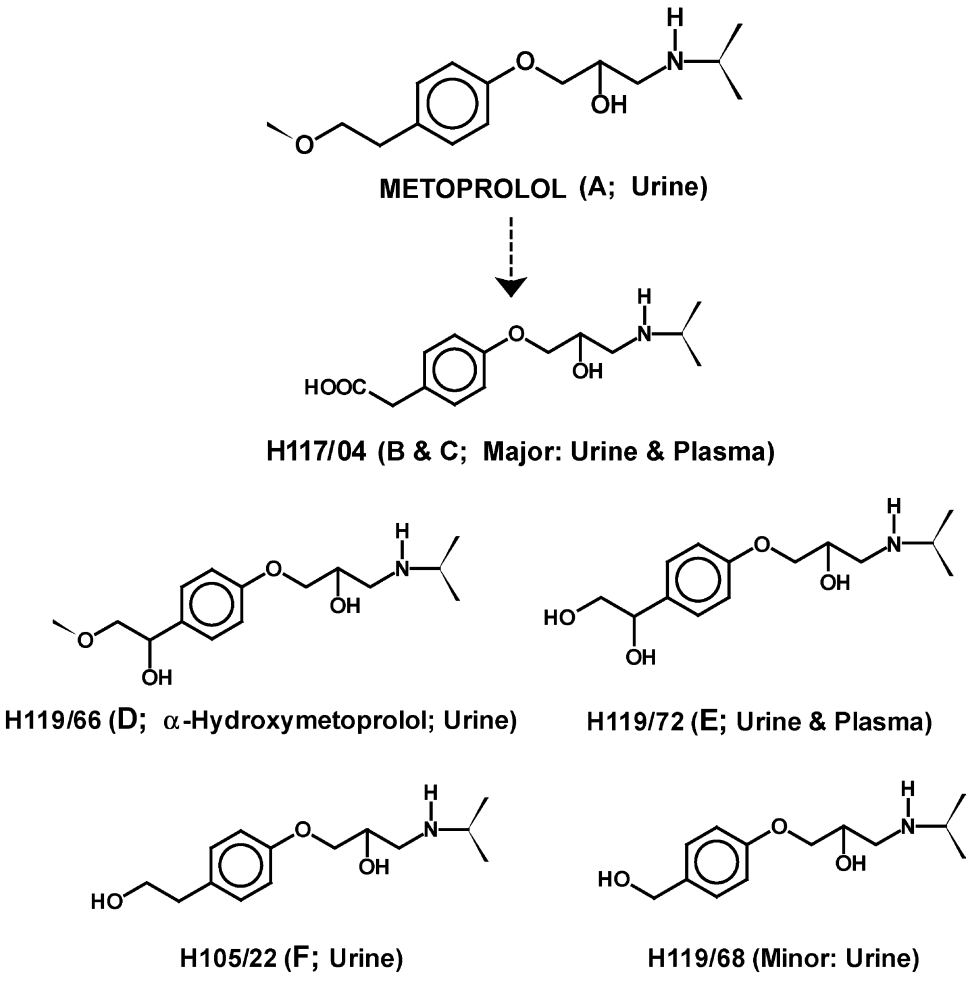

Fig. 1. Structures of metoprolol and its in vivo metabolites identified in horse urine and blood.

raphy (HPLC) with fluorescence detection [14,15]. Methylation of the acid metabolite H117/04 with BF_3 in methanol prior to quantification by HPLC has also been reported [16].

A number of methods for the detection of β-antagonists and their metabolites in human and equestrian sports have been published [17–22]. GC/MS methods are normally used for the screening and confirmatory analysis of basic drugs in horse racing. In vivo metabolism studies are conducted to identify the major metabolite(s) and determine the excretion or detection period of the drug or metabolite(s) at the stated dose and route. The purpose of this study was to identify suitable analytes of metoprolol in horse plasma and urine after administration of a single oral dose. The parent drug and five basic and bifunctional metabolites were isolated by SPE, derivatised as TMS ethers and identified by GC/MS. Their structures are shown in Fig. 1 and their nomenclature is adapted from [6], which also describes their syntheses. Using the standard SPE method for basic drugs [21], an unexpected methylation (Fischer esterification) of the carboxyl group of the major zwitterionic metabolite H117/04 by methanol was observed [23]. The present paper describes the results of these studies.

2. Experimental

2.1. Reagents and chemicals

Glass distilled grade organic solvents were purchased from Rathburn Chemicals Ltd., (Peebleshire, UK). Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and β-glucuronidase from *Helix pomatia* (G 7017, type HP2; mixed β-glucuronidase and aryl sulfatase enzymes) were obtained from Sigma-Aldrich Co. Ltd., (Poole, UK). XtracT® mixed-mode SPE cartridges (XRDAH506, 500 mg, 6 ml; Worldwide Monitoring, PA, USA) were obtained from Anachem (Bedfordshire, UK). Metoprolol tartrate (Betaloc tablets, 100 mg each; Astra Pharmaceuticals Ltd., Hertfordshire, UK) was obtained from a local chemist. The authentic metabolites (±)-4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetic acid (H117/04) and (±)-1-[4-(2-hydroxy-3-

isopropylaminopropoxy) phenyl]-1,2-ethandiol (H119/72) were gifts from AB Hässle (Mölndal, Sweden).

2.2. Drug administration to animals, collection and storage of biofluids

Pre-administration urine samples were collected from two castrated male thoroughbred racehorses (ages: 3 and 6 years; bodyweights: 450 and 500 kg, respectively). Following overnight fasting, a pre-administration blood sample (25 ml) was collected. Metoprolol tartrate (Betaloc, 100 mg = 81.8 mg free base) was administered orally with food. Post-administration blood samples were collected hourly up to 7 h, centrifuged (2000 × *g*, 15 min), the plasma aspirated and stored at −20 °C. The animals were kept in metabolism stalls and all individual urine voids were collected for the up to 72 h. Void times and volumes were recorded and aliquots from each urine sample were stored at −20 °C until analysis.

2.3. Sample pre-treatment and isolation of metabolites

2.3.1. Preparation of unhydrolysed plasma and urine samples

Blank and sequential post-administration samples (5 ml) were adjusted to pH 5.8–6.0 with HCl (2.0 M).

2.3.2. Preparation of urine samples fortified with authentic reference standards

Blank urine samples (5 ml) adjusted to pH 5.8, were fortified with authentic reference compounds metoprolol, H117/04 and H119/72, each at a concentration of 100 ng ml^{-1}.

2.3.3. Preparation of hydrolysed urine samples

Blank and sequential urine samples (5 ml) were adjusted to pH 5 and incubated at 50 °C for 2 h or overnight at 37 °C with *Helix pomatia* enzymes (50 μl). After hydrolysis the samples were readjusted to pH 5.8.

2.3.4. Extraction of biofluids

The prepared samples were centrifuged at 2000 × *g* for 15 min and extracted off-line as described previously [21]. Briefly, the supernatant (5 ml) was applied on mixed mode SPE (XTRACT®) columns primed with methanol (4 ml) and phosphate buffer (0.1 M, pH 6.0; 4 ml) and drawn through the columns at about 1 ml/min. The columns were rinsed with acetic acid (1 M; 2 ml), dried under full vacuum suction for 5 min, the neutral and acidic materials removed by methanol wash (6 ml) and re-dried for a further 3 min. The retained basic and bifunctional metabolites were eluted with ethyl acetate:concentrated ammonia mixture (98:2, by vol.; 5 ml). The solvents were removed under oxygen free nitrogen (OFN) at 60 °C and the residue derivatised as TMS ethers (BSTFA: toluene, 1:1, v/v, 50 μl; 80 °C, 0.5 h). After removal of the derivatisation reagents, the dry residues were dissolved in toluene (50 μl) and analysed by GC/MS.

2.3.5. Modification of normal SPE method

To confirm on-column, acid catalysed esterification of H117/04 by alcohols, the normal methanol wash step described above was substituted with an ethanol wash.

2.4. Capillary column gas chromatography/mass spectrometry

GC/EI+/MS at 70 eV was performed on a Fisons MD800 bench top mass spectrometer interfaced to a Thermoquest Trace 2000 series GC fitted with a SGE BPX5 column (approximately 25 m, 0.22 mm i.d., 0.25 μm film thickness) and a CTC A200S autosampler. The initial temperature of 90 °C was maintained for 1 min, programmed at 15 °C min^{-1} to 320 °C and then maintained at 320 °C for 6 min. The injector, transfer line and the ion source temperatures were 260, 280 and 150 °C, respectively. Aliquots (1 μl) of the derivatised isolates were injected in the splitless mode (1 min) and full scan EI+ mass spectra were recorded from 8 to 20 min of run time by scanning from 50 to 650 Da. TMS derivatised authentic reference standards (10 ng μl^{-1}) were injected with each batch.

Chemical ionisation MS with ammonia as the reagent gas was performed on a Finnigan MAT TSQ 700 instrument, interfaced to a Varian 3400 GC fitted with a SGE BPX5 column as described above. The injector, transfer line and ion source temperatures were 250, 320 and 150 °C, respectively. Spectra were recorded from 8 to 20 min of run time by scanning from 50 to 650 Da. For GC/tandem MS, argon was used as the collision gas (1 mTorr) and product ion spectra were recorded at a collision energy of −10 eV.

3. Results

The structures of metoprolol and its metabolites detected in this study are shown in Fig. 1. The EI+ mass spectra of these compounds are dominated by the charge retained α-cleavage ion *m/z* 72 and provide minimal molecular weight information. Other diagnostic ions are *m/z* 101, *m/z* 188, loss of a methyl group ($M^{+\bullet} - 15$) and the rearrangement ion at ($M^{+\bullet} - 116$) formed by loss of the TMS–O–CH=CH_2 fragment [24]. The reconstructed ion chromatogram (EI+ mode) and the mass fragmentogram of the ion *m/z* 72 (magnification X6 to show the minor peaks), obtained from a derivatised hydrolysed urine extract are shown in Fig. 2. The EI+ and CI+ mass spectra of peaks A, B, D and E are shown in Figs. 3 and 4, respectively. Peak A (Fig. 2; Rt ≈ 11.79 min) was identified as metoprolol (*O*-TMS-ether derivative) by comparison of its EI and CI spectra [Fig. 3(A) and Fig. 4(A), respectively] with those of the authentic drug.

The EI mass spectrum [Fig. 3(B)] of the TMS derivative of the major peak B (Fig. 2; Rt ≈ 12.4 min) showed a

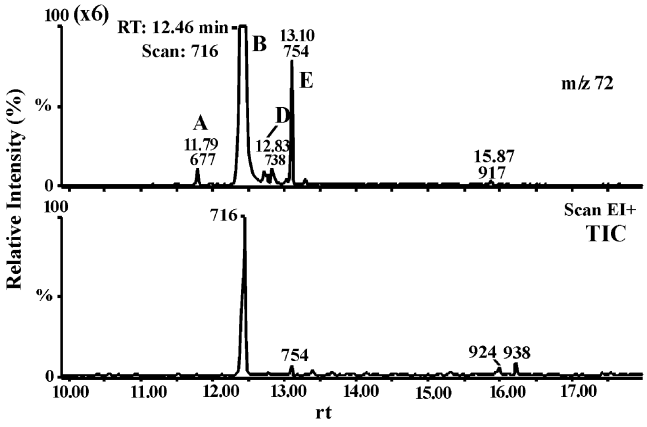

Fig. 2. The total ion chromatogram and the mass chromatogram *m/z* 72 indicating the presence of some basic and bifunctional metabolites of metoprolol in the TMS derivatised isolate of a hydrolysed post-administration urine, extracted by the normal method.

derivatised molecular ion M^+ *m/z* 353 with diagnostic ions at *m/z* 338 ($M^{+\bullet} - 15$), *m/z* 237 ($M^{+\bullet} - 116$), *m/z* 188, *m/z* 101 and *m/z* 72 (base peak). The CI spectrum [Fig. 4(B)] showed a protonated $[M + H]^+$ ion *m/z* 354 indicating an increase of 14 Da on the derivatised M^+ of metoprolol. The product ion spectrum (not presented) showed a base peak at *m/z* 205 $[M + H - (90 + 59)]^+$, major fragment ion *m/z* 145 (probable loss of acetic acid from *m/z* 205) and minor ions at *m/z* 188, *m/z* 98 and *m/z* 72 suggestive of the presence of a *p*-methyl ethanoate group on the phenyl ring. This compound was therefore identified as the mono-TMS derivative of (±)-4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetic acid methyl ester and has never been identified as an in vivo metabolite of metoprolol in any species.

The EI mass spectrum of the TMS ether derivative of a minor peak [Fig. 3(C); Rt ≈ 12.7 min] showed a weak M^+ at *m/z* 411 with fragment ions at *m/z* 396 ($M^{+\bullet} - 15$), *m/z* 295 ($M^{+\bullet} - 116$), *m/z* 188, *m/z* 101 and *m/z* 72 (base peak) and its CI spectrum [Fig. 4(A)] showed the $[M + H]^+$ ion at *m/z* 412. From a comparison of its EI and CI spectra with those of the bis-TMS derivative of authentic H117/04, this metabolite was identified as (±)-4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetic acid. It has been identified as the major metabolite of metoprolol in several species [4–6] and also of betaxolol {(±)-1-(isopropylamino)-3-[*p*-(cyclopropylmethoxyethyl)phenoxy]-2-propanol} in man, formed by elimination of the cyclopropylmethyl group and subsequent oxidation to the carboxylic acid [25].

The EI mass spectra of the TMS derivatives of authenticH117/04 extracted from fortified urine by (1) normal SPE, (2) modified SPE with ethanol wash and (3) unextracted standard are shown in Fig. 5 and demonstrate the methylation [Fig. 5(1)] and ethylation [Fig. 5(2)] of the carboxyl group. The CI mass spectra of the metabolites of metoprolol isolated from urine by the modified method are shown in Fig. 6. Except for spectrum B [Fig. 6(B); Rt ≈ 12.71 min], where the $[M + H]^+$ ion increased by 28 Da to *m/z* 368, all other spectra remained unchanged, confirming the acid catalysed on-column esterification of the carboxylic acid group of H117/04 by alcohols. From this evidence, peak B (Fig. 2) was confirmed as the mono-*O*-TMS derivative of the methyl ester of H117/04. This compound is therefore formed ex vivo as an artefact, from on-column (Fischer) esterification by alcohols at the wash step. It was the only compound detected in hydrolysed urine by GC/MS for about 60 h after oral administration of metoprolol.

The EI and CI mass spectra of compound D [Rt ≈ 12.85 min; Fig. 3(D) and Fig. 4(D)] were identical to those of

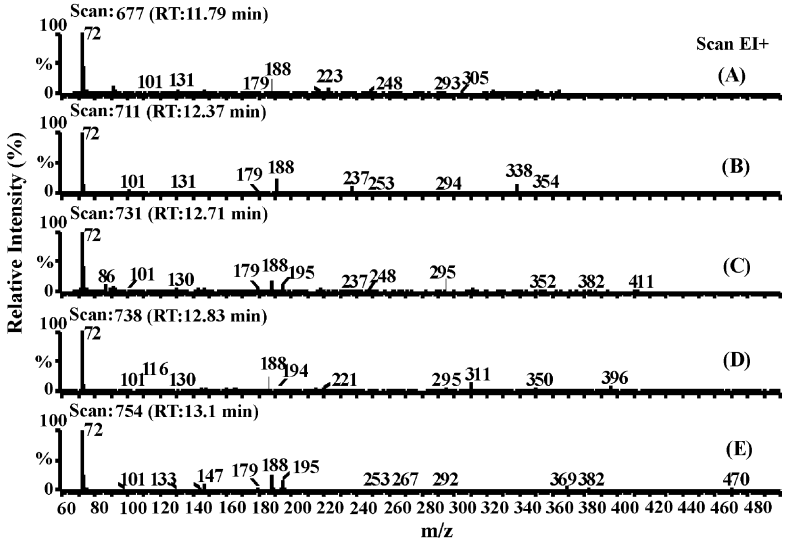

Fig. 3. EI+ mass spectra of (A) metoprolol (as *O*-TMS), (B) (±)-4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetic acid methyl ester (as *O*-TMS), (C) H117/04 (as bis-TMS), (D) α-hydroxymetoprolol (as bis-*O*-TMS) and (E) (±)-4-[(2-hydroxy-3-iospropylaminopropoxy)phenyl]-1,2-ethandiol (H119/72, as tris-*O*-TMS) detected in a hydrolysed urine extract (normal method).

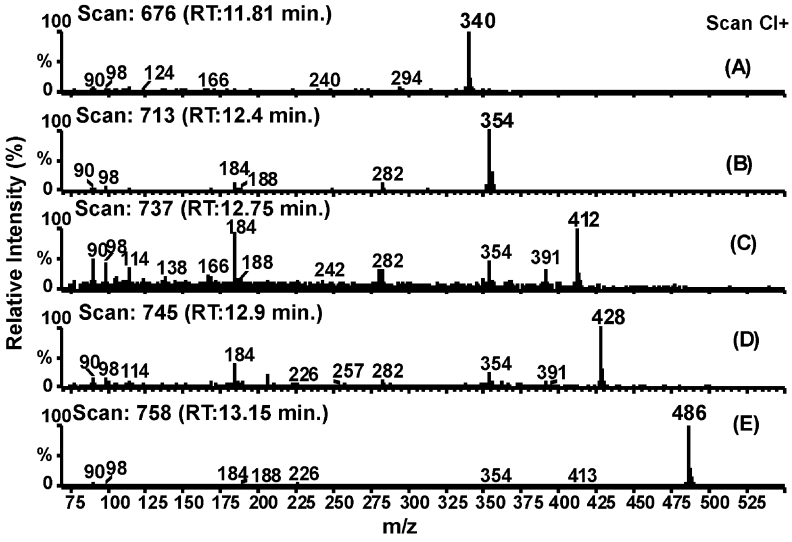

Fig. 4. The CI+ mass spectra of the TMS derivatives of (A) metoprolol, (B) methylated H117/04, (C) H117/04, (D) α-hydroxymetoprolol and (E) H119/72 detected in the hydrolysed urine extract (normal method).

the bis-TMS derivative of α-hydroxymetoprolol {(±)-1-(isopropylamino)-3-[4-(1-hydroxy-2-methoxyethyl)phenoxy]-2-propanol; H119/66}. This metabolite has been identified in man, dog and rat [4] and is a minor metabolite in horse urine. The EI and CI mass spectra of compound E [Rt ≈ 13 min; Fig. 3(E) and Fig. 4(E)] were identical with those of the tris-TMS ether derivative of the authentic standard H119/72. This metabolite was therefore identified as (±)-1-[4-(2-hydroxy-3-isopropylaminopropoxy)phenyl]-1,2-ethandiol. It has been detected from in vitro and in vivo studies in rat and man [4–6] and is the second major metabolite of metoprolol detected for up to 36 h in hydrolysed urine.

The CI mass spectrum of another minor metabolite excreted in horse urine [Fig. 6(F)] shows a protonated $[M+H]^+$ ion at *m/z* 398. The EI spectrum (not presented) showed a base peak at *m/z* 72 and diagnostic ions at *m/z* 382 ($M^{+\bullet}-15$) and *m/z* 281 ($M^{+\bullet}-116$). From these data, this compound was identified as the bis-TMS ether of (±)-1-(isopropylamino)-3-[4-(2-hydroxyethyl)phenoxy]-2-propanol (H105/22) and has been previously identified in urine of man and rat [4–6].

The EI spectrum of a very minor peak detected in urine extracts showed a base peak at *m/z* 72 with minor characteristic ions at *m/z* 188, *m/z* 267 ($M^{+\bullet}-116$) and *m/z* 368 ($M^{+\bullet}-15$). The CI spectrum showed a $[M+H]^+$ ion at *m/z* 384. Based on these data (spectra not shown), the structure (±)-1-(isopropylamino)-3-[(4-hydroxymethyl)phenoxy]-2-propanol (H119/68; as bis-TMS derivative) was assigned to this metabolite. It has also been previously identified in man and rat [4–6].

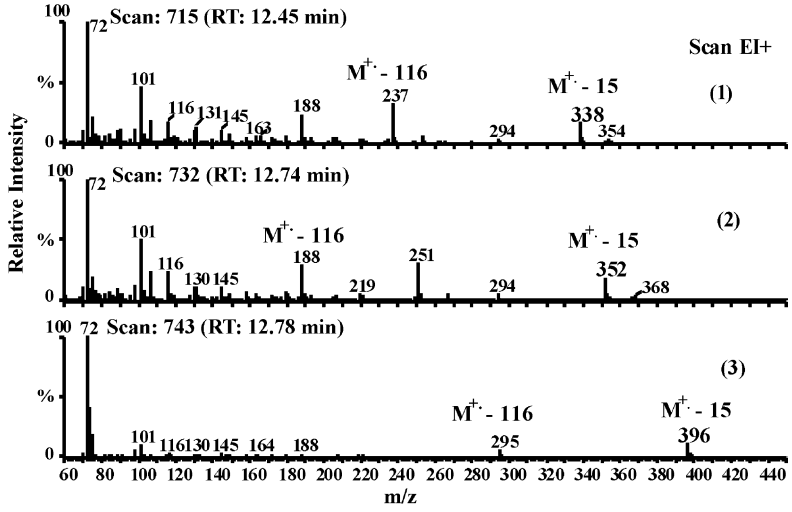

Fig. 5. EI+ spectra of the TMS derivatives of authentic H117/04 after isolation from fortified urine using (1) normal, (2) modified extraction methods and (3) the unextracted standard, confirming artefact formation by on-column alkylation of COOH moiety by alcohols during SPE.

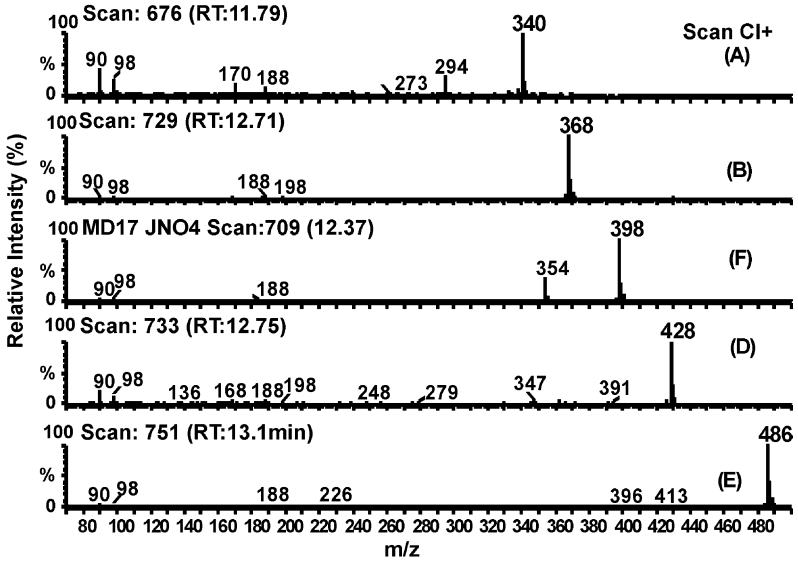

Fig. 6. The CI+ mass spectra of the TMS derivatives of (A) metoprolol, (B) ethylated H117/04, (D) α-hydroxymetoprolol, (E) H119/72 and (F) detected in the hydrolysed urine extract (modified method, ethanol wash).

Only two unconjugated compounds derived from orally administered metoprolol were detected in plasma (data not given). Compound B, the methylated artefact of H117/04 (as mono-TMS), was detected in all samples obtained up to 7 h post-administration; peak concentration was observed at 4 h. The minor ethanediol metabolite E (H117/72) was present at low levels only in samples collected between 3 and 6 h post-administration. Neither unconjugated drug nor any other metabolites were detected in plasma.

4. Discussion

The metabolism and detection of a number of β-adrenergicmimetics in man and animal species have been reviewed [26,27]. The in vivo biotransformation of metoprolol tartrate in thoroughbred horse was studied for the first time after administration of a single oral dose. The metabolites were isolated using mixed-mode SPE cartridges and analysed as their TMS ether derivatives by EI, ammonia CI and tandem MS. For structure elucidation, data were compared where available, with authentic reference compounds and literature. Metoprolol and five basic and bifunctional compounds were detected. Biotransformation of metoprolol in the horse (Fig. 1) is very similar to that of man, rat and dog [4–6]. It is confined to the oxidative transformation of the *p*-(2-methoxy)ethyl substituent. Both, the zwitterionic metabolite H117/04 (major) and H119/72 (compound E, Fig. 1), the second major metabolite were detected in plasma and urine. Phase II biotransformation of metoprolol and its metabolites has not been observed in any species [4]. However, in the present study, analysis of post-administration urine samples before and after hydrolysis with *Helix pomatia* provided indirect evidence of conjugation with increase in the detection periods of the key analytes. In unhydrolysed urine, metoprolol and H117/04 were detected for 10 and 40 h, respectively, increasing to 15 and 60 h after enzyme hydrolyses. The other minor metabolites were detected only in urine for about 24 h. These metabolites can be used as analytes of choice for the GC/MS detection and confirmatory analysis of metoprolol in post-race horse biofluids.

Metabolite H117/04 identified as the major in vivo metabolite of both metoprolol and betaxolol is reported to be difficult to isolate from biological fluids by liquid or solid phase extraction (SPE) methods [3,25]. In this study, this compound was readily isolated from plasma and urine using mixed mode SPE. In addition, an unexpected solid phase acid-catalysed (Fischer) esterification of the carboxyl moiety by alcohols was observed. Similar on-column methylation of the timolol acid metabolite *N*-[-{4-(3-*tert*-butylamino)-2-hydroxypropoxy}-1,2,5-thiadiazol-3-yl]-*N*-(2-hydroxyethyl) gycine formed by oxidative cleavage of the morpholine ring was also observed (unpublished data).

Silica based mixed mode SPE cartridges [copolymeric (XtracT®) and mixed bed (Certify)] are used to selectively isolate basic drugs from aqueous biological matrices at pH 5.8 to 6.0 using dual non-polar and cation-exchange retention mechanisms. Neutral and unionised acidic compounds are primarily retained on the reverse phase (C8, C18) by non-polar (hydrophobic) interactions with some polar interaction due to residual silanols. The cation exchange phase (sulfonic acid group) is negatively charged over the entire pH range. Depending on the pK_a, at pH 5.8 to 6 most basic compounds exist in solution as their conjugate acid form (ionised) allowing interaction with the cation exchange sorbent. During the acetic acid wash step, the cationic amines are activated and selectively bound by ion-exchange interaction and, at

acid pH, the carbonyl oxygen of the aminoacid metabolite is probably protonated. After thorough drying by vacuum suction, the neutral and acidic compounds including deaminated metabolites of metoprolol, retained by hydrophobic interactions, are removed by the methanol wash. Also at this step, a nucleophillic attack of the alcohol(s) on protonated carbonyl group probably results in the formation of a tetrahedral carbonyl addition intermediate leading to Fischer esterification at room temperature. The equilibrium of the reaction is driven by the use of a large excess of the alcohol as well as the rapid azeotropic removal of water. However, alcohols will not alkylate other functional groups such as phenols, amines and thiols. The analytes retained by ion exchange mechanism are then recovered by displacement with basified (pH 11) organic solvents, dried, derivatised and analysed by GC/MS.

A number of reagents are available for alkylations of the functional groups and heteroatoms of drugs and metabolites in urine or blood. Although the present observation could be useful for the isolation and selective esterification (Fischer type) of known aminocarboxylic acids, it should be regarded as a potential source of artefact formation when using mixed mode (cation exchange) SPE columns for studies on basic drugs capable of biotransformation to aminocarboxylic acid (zwitterionic) metabolites.

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