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The characterisation of the major metabolite of salmeterol in the dog

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Abstract

Salmeterol xinafoate is the first of a new class of long acting, selective β_2-adrenoceptor agonists introduced for the treatment of asthma [1,2]. The major metabolite of salmeterol in the dog has been identified as the 3-catechol sulphate of the benzoic acid derivative. This metabolite was isolated from dog bile and was shown to have very similar physiochemical properties to a major endogenous component of bile, the bile acids, creating a complex analytical challenge. Initial experiments, involving hydrolysis with the enzyme sulphatase, suggested that the metabolite was a sulphate conjugate. However, complete identification of the metabolite was complicated in part due to the loss, by metabolism, of deuterium atoms added to the compound, specifically as a marker for mass spectrometry. Subsequently, a synthesis of salmeterol was completed with deuterium labels in different positions. This material was used as a substrate for dog liver slices, a simpler matrix than dog bile, which provided the basis for the metabolite's identification. The metabolite was characterised by the use of spectroscopic techniques, in particular LC/MS, LC/MS/MS and NMR. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Salmeterol; Metabolism; Dog; Liquid; Chromatography mass spectrometry; Sulphate conjugate

1. Introduction

Salmeterol xinafoate is a new long acting β_2-adrenoceptor agonist introduced for the treatment of asthma. The route of administration of salmeterol in clinical use is via inhalation. It is recognised, however, that much of an inhaled dose of a drug will be swallowed [3] and can be absorbed into the systemic circulation from the gastrointestinal tract. Consequently, the oral route was one of the major routes used for the safety evaluation of salmeterol.

The major route of elimination of drug related material in all species is via the faeces, however, significant species differences were evident in the

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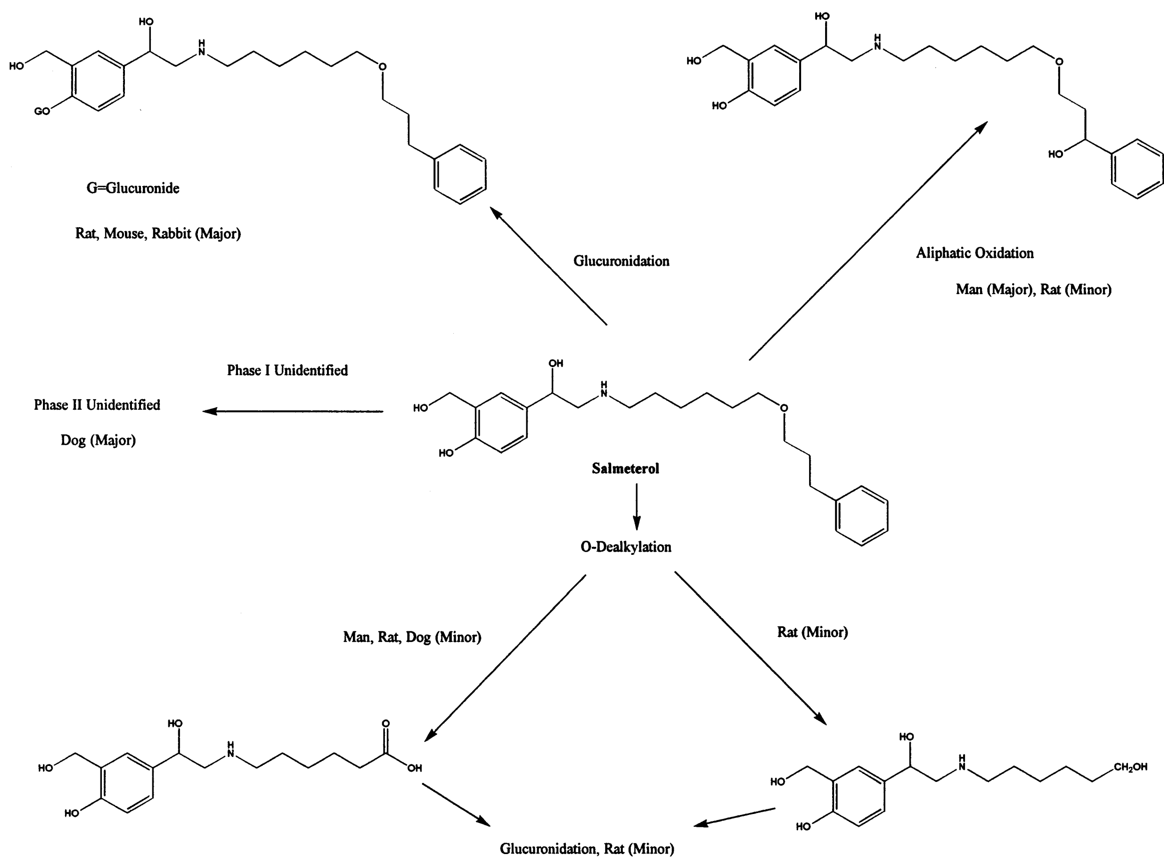


Fig. 1. The metabolism of salmeterol in laboratory animals and man.

metabolism of salmeterol [4]. In man, salmeterol undergoes aliphatic oxidation, whereas in rat, rabbit and mouse, the predominant metabolic route is glucuronidation of the unchanged drug (Fig. 1). Studies in bile duct cannulated dogs indicated that the drug related material in the faeces is principally derived from biliary excretion of metabolites. Analysis of the dog bile, by liquid chromatography with radiochemical detection, showed that the drug related material consisted predominately of one metabolite.

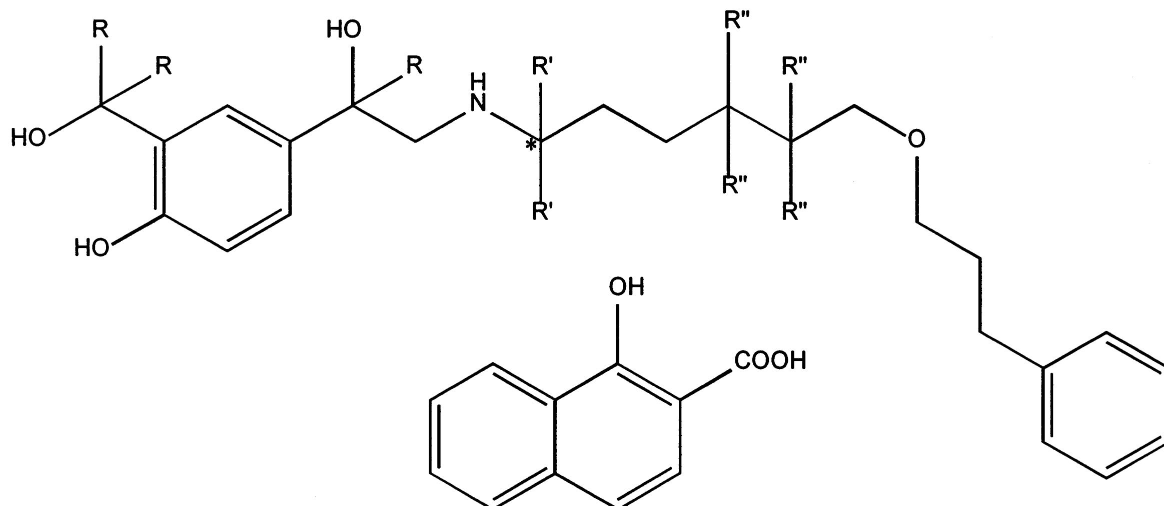
This paper describes the characterisation of the major dog bile metabolite, achieved by spectroscopic techniques in combination with both in vitro and in vivo studies, using isotopically labelled material.

2. Materials and methods

2.1. Chemicals and reagents

Salmeterol hydroxynaphthoate (I), [^{14}C]salmeterol hydroxynaphthoate (II), [$^{2}H_{2}$]salmeterol hydroxynaphthoate(III), [$^{2}H_{3}$]salmeterol hydroxynaphthoate (IV) and [$^{2}H_{4}$]salmeterol hydroxynaphthoate(V) were synthesised by Glaxo Wellcome (Ware, Herts, UK) (Fig. 2).

Citric acid (Analytical Reagent Grade) and trifluoroacetic acid (Analytical Reagent Grade) were supplied by BDH (Poole, Dorset, UK). Acetonitrile (HPLC Grade) and methanol (HPLC Grade) were supplied by Rathburn (Walkerburn, Scotland, UK).



R=R'=R"=H, **Salmeterol hydroxynaphthoate (I)**

R=R'=R"=H * = ^{14}C (II)

R'=^{2}H, R=R"=H (III)

R=^{2}H, R'=R"=H (IV)

R"=^{2}H, R=R'=H (V)

Fig. 2. Salmeterol hydroxynaphthoate used in the in vitro and in vivo experiments.

2.2. Animal experiments

In the first experiment, a male beagle dog, fitted with an indwelling biliary fistula, received a single oral dose of salmeterol hydroxynaphthoate as a mixture (1:1, w/w) of (I) and (IV) with a trace amount of (II), at a dose level of 0.25 mg kg^{-1}. In the second experiment, a male beagle dog, fitted with an indwelling biliary fistula, received a single oral dose of salmeterol hydroxynaphthoate as a mixture (1:1, w/w) of (III) and (V) with a trace amount of (II), at a dose level of 0.25 mg kg^{-1}. Samples of bile were collected upto 8 h post-dose.

2.3. Dog liver slice incubations

Dog liver slices were prepared using a Krumdieck tissue slicer (Alabama Research and Development, USA) and were produced with a thickness of between 250 and 300 µm. The slices were incubated at 37°C, in Williams Medium E Buffer, with a mixture (1:1, w/w) of the deuterated analogues of salmeterol (III) and (V), at a substrate concentration of 25 µM using a roller culture system [5]. Samples of the supernatant from the incubation were analysed by liquid chromatography mass spectrometry (LC/MS) and liquid chromatography tandem mass spectrometry (LC/MS/MS).

Table 1
Proposed assignments for the ions in the product ion mass spectrum, *m/z* 528 of the dog liver slice metabolite, eluting at retention time 21.2 min

Ion (*m/z*)	Proposed assignment
510	$[M+H-H_2O]^+$
448	$[M+H-SO_3]^+$
430	$[M+H-SO_3-H_2O]^+$
412	$[M+H-SO_3-2H_2O]^+$
282	
264	*m/z* 282 $-H_2O$
246	*m/z* 282 $-H_2O$
149	
107	

2.4. LC/MS and LC/MS/MS

A Perkin Elmer Sciex API-3$^+$ mass spectrometer (PE Sciex, Concord, Ontario), equipped with the manufacturer's ionspray source, was used for the analyses. The mass spectrometer was operated in both positive and negative ion, full scan, modes as well as the product ion mode for MS/MS. The orifice voltage was maintained at 70 V and argon, at a collision gas thickness of 250 (equivalent to approximately 400×10^{12} molecules cm^2) was used for MS/MS operation. Data were acquired and processed using standard Perkin Elmer Sciex software on an Apple Macintosh Quadra 950 computer.

A Hewlett Packard HP1090M LC system (Hewlett Packard, Winnersh, UK) equipped with an autoinjector was used for the LC/MS and LC/MS/MS analyses. A diode array detector that was part of the HP1090 LC system, equipped with a standard flow cell, was placed in-line with the mass spectrometer. The detector was set to moni-

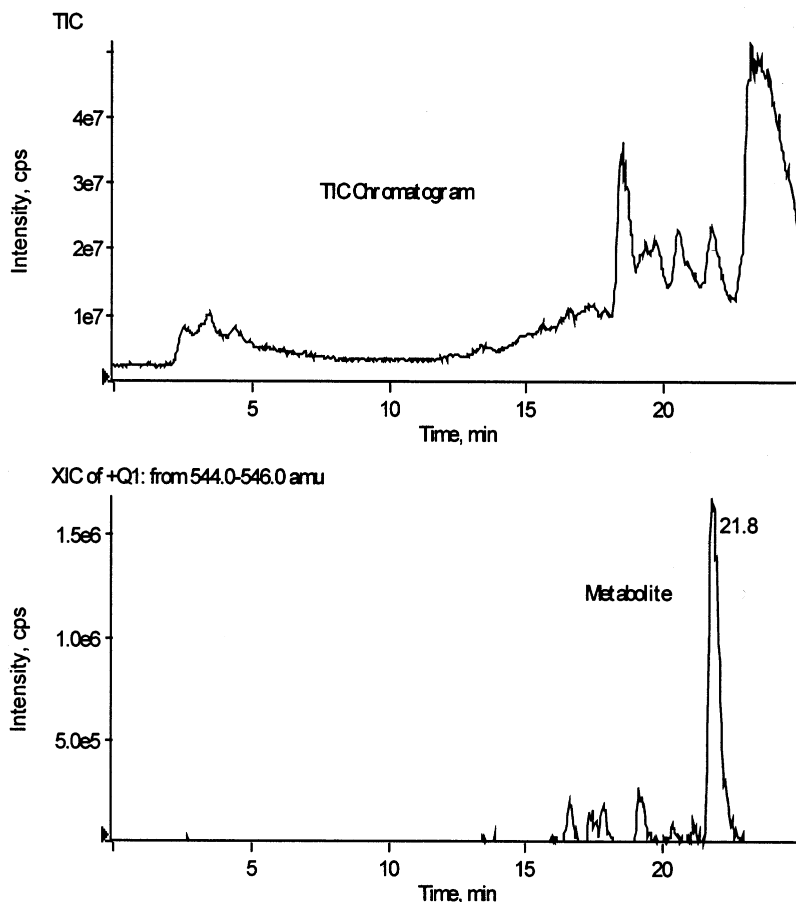


Fig. 3. Chromatograms obtained from the analysis of bile following administration of deuterated salmeterol to the dog.

tor the response at 276 nm with UV spectra acquired over the range 190–600 nm. The analytical column used in these analyses was an Inertsil ODS2 (5 μm, 150 × 1.0 mm, Capital HPLC, Broxburn, Scotland, UK). The mobile phase consisted of A-Water + trifluoroacetic acid (99.98: 0.02, v:v) and B-acetonitrile + trifluoroacetic acid (99.98:0.02, v:v) delivered as a gradient: 0–2 min, 15% Solvent B; 2–20 min, 15–60% Solvent B. The flow rate was maintained at 50 μl min^{-1}.

2.5. Isolation of the metabolite for NMR

A sample of bile (2–4 h, 50 μl) was diluted with water (1:19, v:v) and applied to a pre-conditioned solid phase extraction (SPE) cartridge (aminopropyl, 100 mg, Bondelut, Varian, UK). The cartridge was washed with water and the drug related material eluted in citric acid (1 M, 2 ml). The citric acid eluate was applied to a second SPE cartridge (C18, 100 mg). The cartridge was washed with water and the drug related material eluted in methanol (2 ml), which was subsequently reduced to dryness under nitrogen. The extract was further purified by HPLC. The HPLC system consisted of a Hewlett Packard HP1050 LC pump (Hewlett Packard, Winnersh, UK) and a Berthold HPLC radioactivity monitor (Berthold Instruments, St. Albans, UK) fitted with a heterogeneous flow cell (1 ml). Injections were made from a Gilson 231 autosampler (Anachem, Luton, UK) onto an Inertsil ODS2 analytical column (5 μm, 150 × 4.6 mm, Capital HPLC, Broxburn, Scotland, UK) in conjunction with a Nova-Pak C18

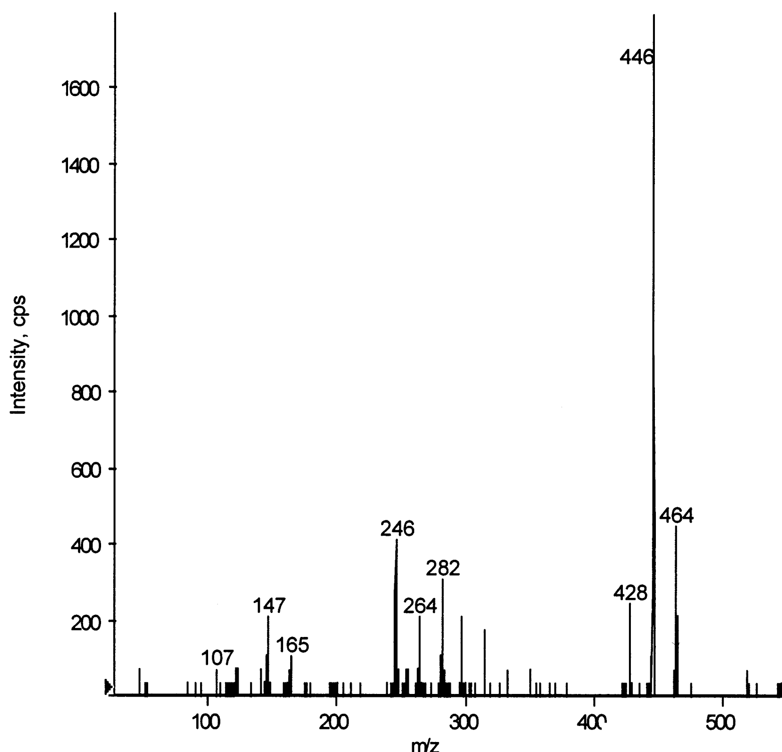


Fig. 4. Ionspray liquid chromatography product ion mass spectrum of the major dog biliary metabolite of salmeterol.

guard column (Waters, Harrow, UK). The mobile phase consisted of A-Water + trifluoroacetic acid (99.98:0.02, v:v) and B-acetonitrile + trifluoroacetic acid (99.98:0.02, v:v), delivered as a gradient: 0–8 min, 15% Solvent B; 8–20 min, 15–60% Solvent B; 20–25 min, 60% Solvent B. The flow rate was maintained at 1.0 ml min^{-1}.

2.6. NMR

NMR spectra were recorded at ambient temperature, at 600 MHz, using a Bruker AMX600 spectrometer. The samples were prepared in deuterated methanol (solution volume, 400 μl). A spectral width of 7246.38 Hz (12 ppm) was used with 32K data points. A total of 4134 transients were acquired using a pulse width of 12 μs (90°) and a 2.26 s acquisition time with a 1 s dual pre-saturation of the residual methanol and HOD signals between pulses giving a repetition rate of 3.26 s.

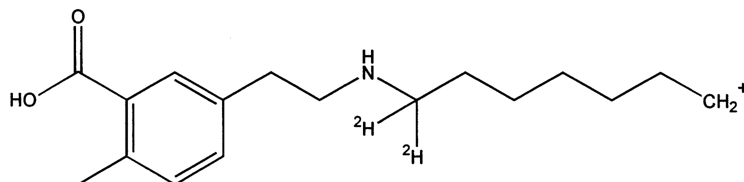
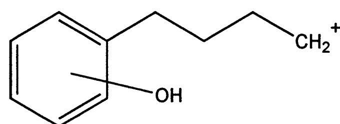
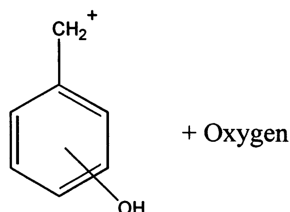
3. Results and discussion

3.1. Initial in vivo experiments with [$^2H_3$]salmeterol

Studies were designed to identify the metabolite of salmeterol in dog bile, using mass spectrometry, by administering a mixture of non-deuterated (I) and deuterated salmeterol hydroxynaphthoate (IV) with a trace amount of carbon-14 radiolabelled material (II). The deuterium labels were positioned at atoms in the molecule that were not expected to be vulnerable to metabolic attack.

A major metabolite was detected by radiochromatographic analysis and subsequent hydrolysis of a bile sample, with aryl sulphatase, suggested that the metabolite was a sulphate conjugate. A variety of solid phase extraction cartridges, with different stationary phases, were investigated to try to purify the metabolite for analysis, whilst at the same time providing information about the functional groups on the molecule. The results

Table 2
Proposed assignments for the ions in the product ion mass spectrum, m/z 544 of the major dog biliary metabolite of salmeterol

Ion (m/z)	Proposed assignment
464	$[M+H-SO_3]^+$
446	$[M+H-SO_3-H_2O]^+$
428	$[M+H-SO_3-2H_2O]^+$
282	
264	m/z 282 $-H_2O$
246	m/z 282 $-H_2O$
165	
147	m/z 165 $-H_2O$
107	

indicated that after the removal of the sulphate group the metabolite still had anionic functionality, unlike salmeterol with cationic functionality.

When samples of extracted dog bile were analysed by ionspray LC/MS, no data could be related to the dosed material by means of the characteristic mass spectral pattern, two ions separated by 3 mass units, in the ratio 1:1. Mass spectral data that were obtained consisted predominately of bile acids.

The hydrolysis and solid phase extraction data suggested that the major dog biliary metabolite of salmeterol was anionic and conjugated as a sulphate, however, extensive chromatographic and LC/MS investigations could provide no further evidence pertaining to the metabolite's structure. These findings also inferred that the carbon-14 radiolabel had not been lost, however, no evidence had been obtained to support the presence of the deuterium atoms. Based on the results from earlier metabolism studies in other species, the deuterium atoms had been located at metabolically less vulnerable sites within the molecule. However, the possibility remained that one, or

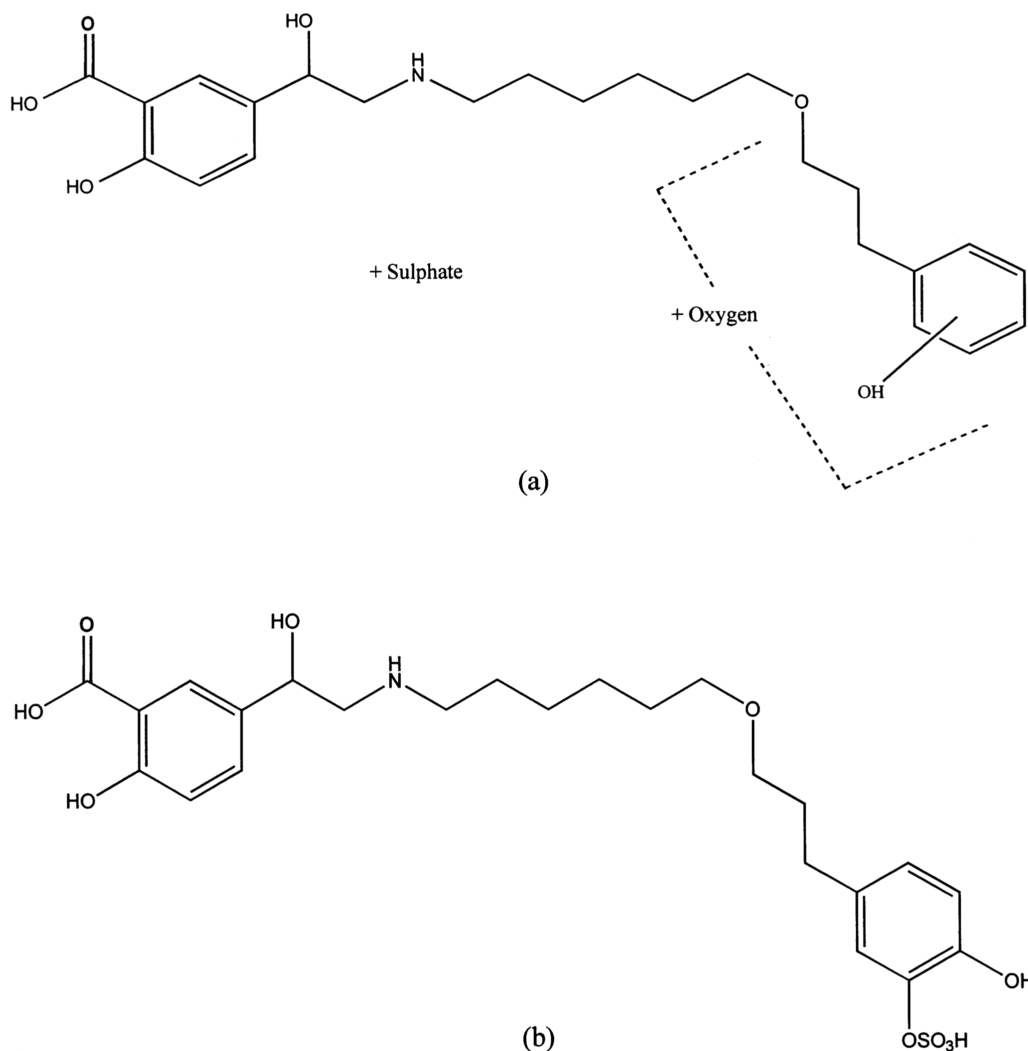


Fig. 5. Structure of the dog bile metabolite proposed from mass spectral data (a) and the definitive structure proposed by nuclear magnetic resonance spectroscopy (NMR) (b).

more, of the deuterium atoms had been removed by metabolism. Metabolism at the sites on the molecule containing the deuterium atoms was further investigated using salmeterol containing deuterium atoms located in different positions on the molecule. These studies were performed using both in vitro and in vivo systems.

3.2. In vitro experiments with [$^2H_2$]salmeterol and [$^2H_4$]salmeterol

A major problem with the identification of the major dog biliary metabolite of salmeterol was that it was thought to be a sulphate conjugate yet its chromatographic properties indicated that its lipophilicity was similar to salmeterol. Bile acids have very similar properties and respond well in ionspray LC/MS which made the purification procedures very difficult [6]. One approach to overcome this problem was the preparation of a sample of the metabolite using an in vitro system and thereby greatly reducing the presence of bile acids. The in vitro system selected was the liver slice system as this contains all the necessary

enzymes and co-factors to perform both Phase I (e.g. oxidative) and Phase II (conjugative) metabolism [7].

A mixture of deuterated salmeterol III and V (1:1, w:w) were incubated with dog liver slices. The supernatant was removed directly from the incubation and analysed by ionspray LC/MS. The chromatograms and mass spectral data obtained showed unchanged salmeterol substrate eluting with a retention time of 22.9 min, with the major ions at m/z 418/420 assigned as $[M+H]^+$. The metabolite eluting with a retention time of 21.2 min has a similar isotopic pattern to that of salmeterol. This metabolite, which is 110 Da greater than salmeterol, corresponds to a metabolite not previously observed.

A sample of the liver slice incubation supernatant was concentrated by SPE and analysed by LC/MS/MS. The assignments from the product ion mass spectrum obtained for the metabolite eluting at 21.2 min are given in Table 1. These data, obtained from the analysis of the dog liver slice incubation, suggested that this metabolite was a sulphate conjugate with oxidation of the benzyl alcohol to benzoic acid and further oxidation in the monosubstituted phenyl ring. The proposed structure was not that of a previously known metabolite.

Oxidation of the benzyl alcohol function to a benzoic acid group would lead to the loss of two of the three deuterium atoms present in the deuterated salmeterol (IV) used in the initial dog in vivo studies. This in turn would remove the expected isotope pattern from the mass spectrum of the metabolite and hence, the ability to 'observe' this drug related material. For these reasons, it was decided to repeat the in vivo study using the same deuterated salmeterol, that was used in the liver slice incubations, to determine if this novel metabolite was indeed the major metabolite in dog bile.

3.3. In vivo experiments with [$^2H_2$]salmeterol and [$^2H_4$]salmeterol

The chromatograms obtained from the analysis of a sample of bile obtained following administration of a mixture of deuterated salmeterol, (III) and (V), to the dog are shown in Fig. 3. No data were obtained for a metabolite with the same molecular weight, 110 Da greater than salmeterol, as that produced by the in vitro liver slice system. However, a metabolite was observed with a molecular weight of 126 Da greater than salmeterol, which would indicate further oxidation had occurred. The product ion mass spectrum obtained for this metabolite in dog bile is shown in Fig. 4 and proposed assignments are detailed in Table 2. The structure proposed for the dog bile metabolite from mass spectral data is shown in Fig. 5(a).

A sample of the dog bile was concentrated and purified more effectively by making use of the structural information provided by mass spectrometry. Comparison of the NMR spectra of the metabolite with that obtained for authentic salmeterol, confirmed the mass spectrometry findings with regard to the oxidation of the benzyl alcohol function to form the benzoic acid group. The evidence for this is the deshielding effects exerted on the aromatic protons as a result of the formation of the carboxylic acid function [8]. The proton orthro to both the benzyl alcohol groups in salmeterol moved from 7.27 to 7.96 ppm. In addition, the spectrum of the metabolite showed that the phenyl ring of salmeterol had undergone double oxidation to form the 3-catechol sulphate of the benzoic acid derivative (Fig. 5(b)).

The NMR spectrum of the metabolite showed an overall loss of two aromatic protons from the phenyl ring of salmeterol observed between 7.12 and 7.22 ppm and the appearance of three new multiplets at 6.78, 6.84 and 7.14 ppm, consistent with the formation of the 3-catechol sulphate. It was also observed that upon standing these peaks disappeared and that a new set of peaks appeared at 6.59, 6.48 and 6.65 ppm. This is consistent with deconjugation of the sulphate group to form the 3-catechol compound and provided additional structural evidence.

4. Conclusion

One of the most difficult analytical challenges is to characterise an unknown metabolite, having

similar physiochemical properties to a major endogenous component, within the biological matrix of which the metabolite is excreted. Salmeterol xinafoate, a selective β_2-adrenoceptor agonist, produced such a metabolite in the dog, a 3-catechol sulphate of the benzoic acid derivative. The identification of the metabolite was complicated due to the loss, by metabolism, of deuterium atoms added to the compound specifically as a marker for mass spectrometry. The subsequent synthesis of salmeterol, with deuterium labels in different positions, and the use of this material as a substrate for dog liver slices, a simpler matrix than dog bile, provided the basis for the metabolite's identification. The metabolite was characterised by the use of spectroscopic techniques, in particular LC/MS, LC/MS/MS and NMR.

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