*Journal of Pharmaceutical & Biomedical Analysis*
Vol. 8, No. 7, pp. 573–576, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00


# Use of $^1$H NMR in the identification of a metabolite of a catecholic cephalosporin excreted in rat bile

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**Keywords**: *$^1$H NMR; biofluid; metabolism; catecholic cephalosporin.*

## Introduction

The synthesis and antibacterial activities of C(7)α-formamidocephalosporins have been reported previously [1]. The most active compounds described, particularly against Gram-negative bacteria, were the 3,4-dihydroxyphenylpiperazinyl analogues and some of these were selected for evaluation *in vivo* including a pharmacokinetic analysis in the rat. During the course of these studies with one such compound — BRL 41897A — a discrepancy between the bioassay and reversed-phase HPLC results was observed in the amount of compound excreted in the bile. Furthermore an additional later-running peak, considerably larger than that of the parent compound was noted on the HPLC trace of post-dose bile samples. High field $^1$H NMR spectroscopy has been shown recently to be of considerable value in the detection and characterization of metabolites in body fluids [2, 3]. Indeed this technique, in conjunction with 2D $^1$H, $^{19}$F COSY NMR, was used successfully in these laboratories to study the excretion of flucloxacillin and its metabolites in rat urine [4]. This paper reports the direct examination of rat bile by $^1$H NMR and the detection and characterization of a metabolite of BRL 41897A using untreated samples. Subsequent purification of the compound by preparative HPLC and structure confirmation studies are also described.

## Experimental

*Biliary excretion studies*

The bile ducts of two 250 g male Sprague–Dawley rats (Hacking and Churchill) were cannulated as described previously [5]. BRL 41897A, which was synthesized within the laboratories of Beecham Pharmaceuticals, was administered intramuscularly at 50 mg kg$^{-1}$ and bile collected over 0–2, 2–4 and 4–6 h periods.

*Assay of bile samples*

The concentration of antibiotic excreted in the bile was measured by bioassay using *Escherichia coli* NCTC 10418 grown in Diagnostic Sensitivity Test Agar (Oxoid) against standards prepared in 0.9% phosphate-buffered saline (pH 7.4). The bile samples were also passed through disposable 0.45-μm filters (Acro LC 13, Gelman) and examined by analytical reversed-phase HPLC. This employed a mobile phase of 0.05 M acetate buffer (pH 5.0) containing 13% acetonitrile pumped at 2.0 ml min$^{-1}$ using a Gelman high pressure pump through a stainless steel C18 μ-Bondapak column (Waters) protected by a guard column packed with CO-pell ODS (Whatman). Filtered bile was introduced through a Rheodyne 7120 injector fitted with a 20-μl loop, and antibiotic was detected and quantified using a variable wavelength UV detector set at 254 nm.

*NMR spectroscopy*

All spectra were obtained on a Bruker AM 400 NMR spectrometer equipped with a $^1$H/$^{13}$C 5-mm dual probe. A sample of neat bile (450 μl) was placed in an NMR tube to which $D_2O$ (50 μl) was added to serve as an internal field lock. The data were acquired over 5.2 kHz into 32 K data points using a single pulse experiment. The large 100 M $H_2O$

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resonance was partially suppressed by a gated irradiation technique using standard Bruker software. The full spectral assignment of BRL 41897A (Tables 1 and 2) was obtained using a 30 mg/0.5 ml $D_2O$ sample. A variety of one- and two-dimensional experiments were performed to complete the full analysis. 2D $^1H$, $^{13}C$ COSY and 2D $^1H$, $^{13}C$ COLOC experiments aided the virtually unambiguous assignment of both the $^1H$ and $^{13}C$ spectra. Both the proton and carbon spectra were complicated by the presence of two rotameric species in approximately 6:1 ratio resulting from restricted rotation, on the NMR time scale, about the formamido bond. Rotamerism was also exhibited by the metabolite. The nuclear Overhauser enhancement (nOe) experiments conducted on the metabolite were performed using a method similar to that described by Sanders and Mersh [6].

**Table 1**
$^1H$ assignments for BRL 41897A and its metabolite

| BRL 41897A proton shift (ppm) | Assignment | Metabolite proton shift (ppm) |
|---|---|---|
| 9.31 | 28 | 9.39 |
| 9.30* | | 9.37* |
| 8.42 | 10 | 8.44 |
| 8.09* | | 8.12* |
| 6.96 | 18 | 7.12 |
| 6.86 | 14 | 7.03 |
| 6.81 | 15 | 6.91 |
| 5.25* | 12 | 5.39* |
| 5.20 | | 5.32 |
| 5.19* | 6 | 5.25 |
| 5.14 | | 5.16 |
| 4.39 | 26A | 4.38 |
| 4.28* | | 4.26* |
| 3.97 | 26B | 3.96 |
| 3.92 | 20 | 3.94 |
| — | 17-OMe | 3.86 |
| 3.61 | 21 | 3.60 |
| 3.45 | 24 | 3.45 |
| 3.36 | 2A | 3.33 |
| 2.99 | 2B | 2.98 |
| 1.14 | 25 | 1.16 |

*Major rotamer resonance.

**Table 2**
$^{13}C$ assignments for BRL 41897A

| Carbon shift (ppm) | Assignment | Carbon shift (ppm) | Assignment |
|---|---|---|---|
| 173.72 | 11 | 124.58 | |
| 173.43* | | 121.92* | 3 |
| 168.14* | 9 | 121.12 | 14 |
| 167.82 | | | |
| 167.21 | 27 | 117.06 | 15 |
| 167.16 | | | |
| 166.31* | 10 | 115.86 | 18 |
| 164.09 | | | |
| 160.74 | $22^1$ | 77.85 | 7 |
| 159.72* | 8 | 65.31 | 6 |
| 159.41 | | | |
| 157.64 | $23^1$ | 59.06 | 12 |
| | | 58.88* | |
| 157.24 | 28 | 44.10 | $20^2$ |
| 153.90 | 19 | 43.98 | 24 |
| 145.73 | 17 | 41.33 | $21^2$ |
| 145.66* | | | |
| 145.19 | 16 | 38.31* | 26 |
| | | 38.13 | |
| 132.63* | 4 | 27.95 | 2 |
| 132.07 | | 27.68* | |
| 128.37* | 13 | 11.95 | 25 |
| 128.07 | | | |

*Major rotamer resonance. Superscripts 1 and 2 may be reversed.

*Preparative HPLC studies*

The system comprised a high pressure pump (Waters M6000), a valve injector (Rheodyne 7125) fitted with a 2000-μl loop, a UV detector fitted with a preparative cell and set at 254 nm and a recorder (Servogor). Separation was effected by isocratic elution at ambient temperature on a Knauer C8 column (Dr Herbert Knauer, K.G.), 250 × 16 mm i.d., 7 m. The mobile phase was as described previously.

A sample of 0–2 h rat bile (1 ml) was loaded onto the column at a flow rate of 2.0 ml $min^{-1}$, after 2 min the flow was increased to 8.0 ml $min^{-1}$ to effect separation. The metabolite-containing fraction (10 ml) was collected, diluted with water (90 ml) and the resultant solution passed through a C18 Sep-Pak (Waters). The cartridge was rinsed with water (5 ml) and the metabolite eluted with 50% aqueous acetone (2 ml). Evaporation of the eluent at reduced pressure provided the purified metabolite (360 μg).

*Susceptibility testing*

The minimum inhibitory concentrations (MIC) of BRL 41897A and its metabolite were determined by serial dilution in 0.05 ml volumes of Iso-Sensitest Broth (Oxoid) in microtitre plates against a final inoculum of $5 \times 10^4$ cfu/well. The plates were incubated at 37°C for 18 h and the MIC taken as the lowest concentration of antibiotic to inhibit visible growth.

## Results and Discussion

The biliary excretion of BRL 41897A in the rat as determined by bioassay and by analytical HPLC is shown in Table 3. The majority of the compound was present in the 0–2 h

**Table 3**
Excretion of BRL 41897A and its metabolite in rat bile, % excreted at time (h)

| Assay procedure | Rat 1 0–2 | 2–4 | 4–6 | Total | Rat 2 0–2 | 2–4 | 4–6 | Total |
|---|---|---|---|---|---|---|---|---|
| (i) Bioassay | | | | | | | | |
| As BRL 41897A | 9.2 | 3.3 | 0.6 | 13.1 | 15.5 | 3.0 | 0.2 | 18.7 |
| (ii) HPLC | | | | | | | | |
| As BRL 41897A | 0.4 | 0.2 | <0.02 | 0.6 | 1.3 | 0.2 | <0.02 | 1.5 |
| As metabolite | 42.8 | 12.5 | 2.4 | 57.7 | 50.2 | 17.3 | 1.2 | 68.7 |

sample, although the concentrations determined by the two methods were considerably different. Bioassay indicated that 10–15% of the dose was excreted as parent compound or free acid, whereas HPLC suggested only 0.5–1.0%. Other peaks were noted in the bile, however, and so both 0–2 h samples plus predose control bile were submitted for analysis by 400 MHz $^1$H NMR spectroscopy. A comparison of the spectra of the two samples revealed the presence of extra signals in the aromatic region (7.0 ppm), and at 8.10 and 9.35 ppm which could be attributable to BRL 41897A and/or a structurally related molecule.

Figure 1 shows an expansion of the low field region (10.0–6.0 ppm) of the 0–2 h bile spectrum with, above it, the spectrum obtained when the bile sample was spiked with authentic BRL 41897A (40 μl of a 5 mg ml$^{-1}$ solution). It can be seen that the major drug-related component, although similar, is not BRL 41897A. However, the experiment does confirm the presence of BRL 41897A from the marked increase in the intensity of the low field resonance of the pair at 9.35 ppm and the high field resonances of the pairs at 8.10 ppm which are the two rotameric responses of the —NH—CHO proton.

Analysis of the $^1$H NMR spectrum of the metabolite obtained by preparative HPLC showed it to be very similar to BRL 41897A but with an additional methoxy resonance at 3.80 ppm and significant chemical shift differences amongst the aromatic protons. An nOe experiment showed unambiguously that the meta-hydroxyl group had been methylated (see

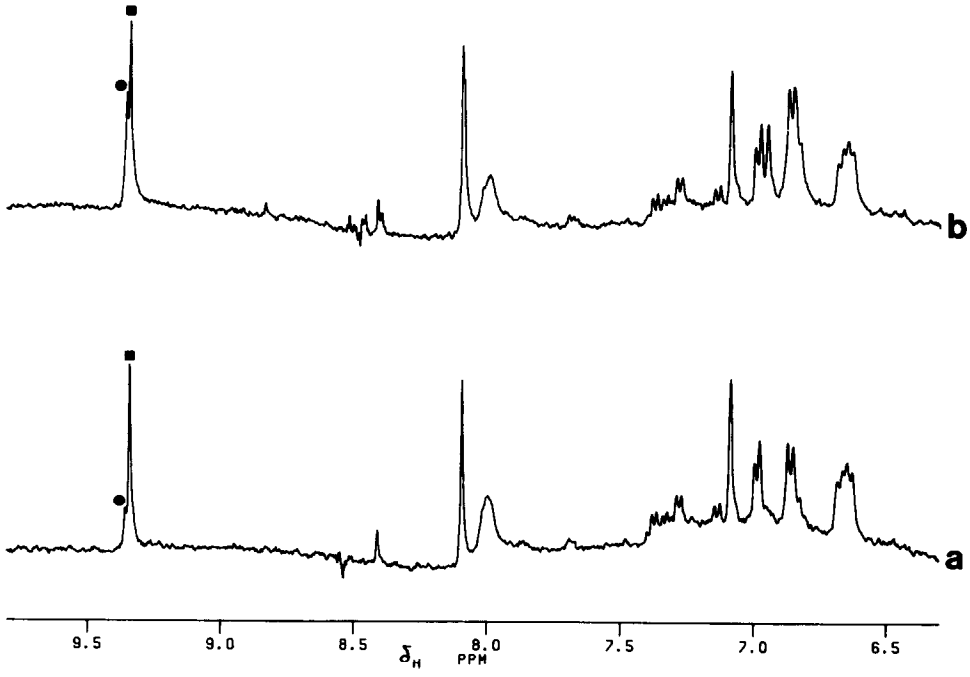


**Figure 1**
(a) The low field region of the 400 $MH_2$ $^1$H NMR spectrum obtained on the 0–2 h bile sample of a rat dosed with BRL 41897A. (b) As (a) but after spiking authentic BRL 41897A into the 0–2 h bile sample. Key: ●, BRL 41897A; ■, metabolite.

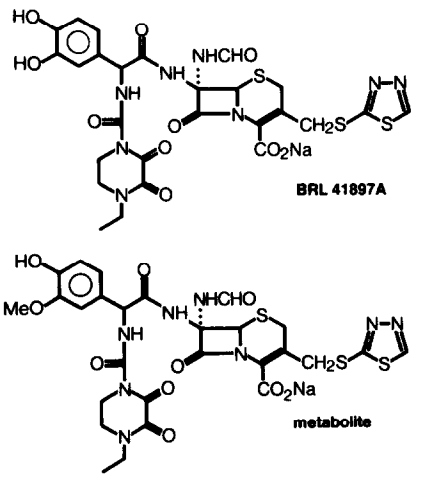


**Figure 2**
Structure of BRL 41897A and methylated metabolite.

Fig. 2). Irradiation of the methoxyl resonance caused an enhancement of only the isolated *ortho*-proton in the nOe difference spectrum.

The purified metabolite was used to quantify the amount present in the original bile samples. Table 3 shows that approximately 60–70% of the dose of BRL 41897A was excreted as metabolite, the majority of which emerged within the first 2 h. The antibacterial activity of the metabolite was also examined. The results in Table 4 show that the compound was from 2 to 64-fold less active than the parent cephalosporin against Gram-negative bacteria although was similar in effect against Gram-positive cocci. The difference in Gram-negative activity between the two compounds explains the spurious bioassay results which were obtained against *E. coli*. The bile sample contained predominantly metabolite, whereas the standards used to quantify the assay were BRL 41897A itself.

**Table 4**
Antibacterial activity of BRL 41897A and metabolite (MIC/μg $ml^{-1}$)

| Organism | BRL 41897A | Metabolite |
|---|---|---|
| *E. coli* DCO | ≤0.03 | 2.0 |
| *E. coli* DCO (RTEM) | ≤0.03 | 2.0 |
| *K. pneumoniae* T767 | ≤0.03 | 2.0 |
| *E. cloacae* N1 | 1.0 | 2.0 |
| *E. cloacae* P99* | 2.0 | 4.0 |
| *P. aeruginosa* NCTC 10662 | 1.0 | 8.0 |
| *P. aeruginosa* Badia* | 0.25 | 8.0 |
| *S. aureus* Oxford | 4.0 | 4.0 |
| *S. pyogenes* CN10 | 0.5 | 0.5 |

*Ceftazidime-resistant strain.

## Conclusion

Proton NMR spectroscopy has been shown to be a powerful tool for the detection and characterization of a catecholic cephalosporin and its methylated metabolite in rat bile. Spectral crowding from endogenous biofluid component resonances proved not to be a problem. Indeed within about a week of the original rat pharmacokinetic study using BRL 41897A, a metabolite was identified by $^1H$ NMR; extracted by preparative reversed-phase HPLC to confirm the structure and its antibacterial activity *in vitro* determined in comparison with that of the parent cephalosporin.

*Acknowledgements* — We are grateful to Mr K. Jennings and Miss H. Smulders for competent technical assistance, and to Dr R. Southgate and Dr J. Everett for helpful advice with the manuscript.

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[*Received for review 2 February 1990*]