Journal of Chromatography B, 911 (2012) 122–132

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

# Metabolism studies of benzbromarone in rats by high performance liquid chromatography–quadrupole time of flight mass spectrometry

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ARTICLE INFO

*Article history:*
Received 14 June 2012
Accepted 25 October 2012
Available online 2 November 2012

*Keywords:*
Benzbromarone
Metabolite
HPLC–QTOF-MS

ABSTRACT

A high performance liquid chromatography–quadrupole time of flight mass spectrometry (HPLC–QTOF-MS) method was employed in investigation of benzbromarone metabolites in rat plasma, urine, feces and bile samples. Meanwhile, the metabolic pathways of benzbromarone in rats were discussed. The identification was achieved on a reversed-phase $C_{18}$ column with mobile phase gradient method. The QTOF-MS was operated under full scan of MS or MS/MS in negative mode. The fragments were acquired by raising collision induced dissociation (CID) energy for speculating the structures of parent ions. According to the information from the chromatograms and mass spectra, 17 metabolites were obtained. Among them, the deoxidized phase I metabolites and an array of phase II metabolites—sulfate conjugates detected in the biological samples made the work more significant.



## 1. Introduction

Gout is caused by high-level uric acid precipitation in the joints or soft tissues, causing joint pain, swelling and inflammation. Benzbromarone (BBR) is benzofuran derivative (as shown in Fig. 1) acting as an uricosuric agent by reducing the proximal tubular reabsorption of uric acid [1]. But recently, clinical cases of acute liver damage related to BBR [2–5] draw our attention to the metabolism profiles of BBR *in vivo*.

Debromination was considered to be a main bioactivation in the metabolic pathways of BBR *in vivo* before late 1980s [6,7]. It was clarified that hydroxylation rather than debromination was the predominant bioactivation of BBR in 1988 [8]. Soon after that, its metabolism study in a healthy volunteer was performed by Amold et al. [9], who determined six hydroxyl metabolites of BBR with comprehensive methods including HPLC, LC–MS and GC–MS. In addition, they pointed out that glucuronide conjugates were the major phase II metabolites with enzymatic hydrolysis method. In 1990s, many people held that the biotransformation of BBR varied in humans through a population study and that the toxicity of BBR might be a familial disorder [10–14]. The only data available on the toxicity of BBR in humans had been published by McDonald and Allan Rettic [1]. They insisted that a metabolite intermediate containing a catechol structure played a key role in its liver toxicity.

Although the metabolic properties of BBR have been examined, to date no systematic researches into its elimination *in vivo* have been presented with a fast and accurate analyzer. Therefore, it is important to develop sample preparation and analysis techniques for screening BBR metabolites in different biological matrices.

Recently, HPLC–QTOF has been proved a powerful and reliable analytical approach for *in vivo* metabolite identification studies. It is a combination of HPLC and QTOF technology for the identification of drug metabolites in biological matrices that adds a new dimension to metabolism studies enabling us to obtain better throughput, faster analysis, increased sensitivity, and increased peak resolution, which in turn will improve the data quality from the mass spectrometer [15,16].

Based upon the literature survey above, the present study aims to develop a rapid, simple and accurate approach to the comprehensive confirmation of BBR metabolites *in vivo* and meanwhile to lay the material foundation for further research on the BBR-induced hepatotoxicity from systematic studies on the plasma, urine, feces and bile samples of the oral dosed rats.

## 2. Experimental

### 2.1. Chemicals and reagents

BBR (>99.9% purity) was synthesized by ourselves; acetonitrile (HPLC grade, Fisher, USA); formic acid (HPLC grade, DIKMA, USA);

*Abbreviations:* HPLC–QTOF-MS, high performance liquid chromatography–quadrupole time of flight mass spectrometry; CID, collision induced dissociation; BBR, benzbromarone.

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http://dx.doi.org/10.1016/j.jchromb.2012.10.035

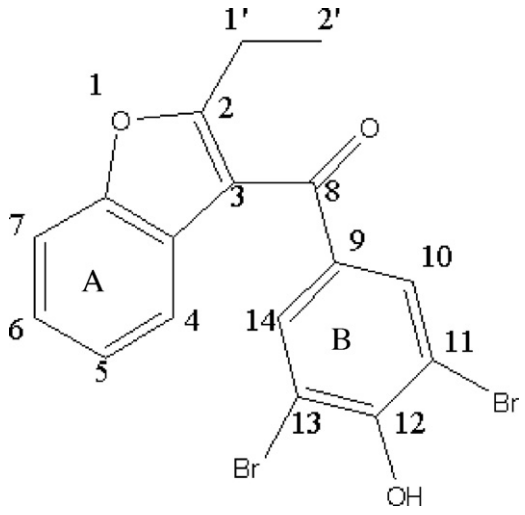


**Fig. 1.** The structure of BBR.

**Table 1**
Gradient of mobile phase for separation.

| *t* (min) | Phase A (%) | Phase B (%) |
|---|---|---|
| 0 | 20 | 80 |
| 20 | 60 | 40 |
| 30 | 90 | 10 |
| 35 | 20 | 80 |

*Note*: *Note:* Phase A: acetonitrile with 0.2% formic acid; Phase B: water with 0.2% formic acid.

other solvents were purchased commercially with purity of analytical or HPLC grade.

### 2.2. Instrumentations

HPLC system (Agilent, USA) consisted of a model G1276A pump, model G1367B autosampler and model G1316A UV-detector. The chromatograph was equipped with a reversed-phase $C_{18}$ column of Waters (4.6 mm × 75 mm, 3.5 μm, USA) eluted with a gradient mobile phase (tabulated in Table 1). The flow rate was 0.8 mL min$^{-1}$ and the column temperature was ambient temperature. The UV detector was set at 235 nm. An accurate splitter was used to split the flow into 1:3 before introduction into MS.

The QTOF-MS system (Bruker, Germany) with an ESI source was performed in negative mode. The parameters of ESI-MS were set as follows: capillary voltage (+3800 V), the nebulizer gas pressure (1.2 bar), the dry gas flow rate (8.0 L min$^{-1}$) and temperature (180 °C). MS conditions were corrected by direct infusion of sodium formic acid solution (1 mM L$^{-1}$) delivered by a syringe pump at a flow rate of 3 μL min$^{-1}$. The data were analyzed by Bruker Daltonics Data Analysis 3.4 software.

### 2.3. Animal handling and sample collection

The investigations were performed on nine male Sprague–Dawley rats (220 ± 20 g) provided by the Department of Experimental Animals of Shenyang Pharmaceutical University (Shenyang, China). Environmental controls for the animal room were set as follows: temperature at 22 ± 3 °C, relative humidity at 55 ± 5%, and a 12-h light/dark cycle. The animal studies were approved by the Animal Ethics Committee of Shenyang Pharmaceutical University and carried out in accordance with the requirements of Chinese national legislation.

The rats were fasted for 12 h prior to the study and allowed water *ad libitum*. The rats were exposed to BBR (25 mg kg$^{-1}$ bodyweight) prepared with 0.5% sodium carboxymethyl cellulose solution (2.0 mg mL$^{-1}$) for a common oral dosing, and then fasted with free access to water.

#### 2.3.1. Plasma sample collection

The blood samples at each time point were collected from three of rats. The samples were respectively collected from the eye bottom into heparinized tubes at the predose, 2, 4, 6, 8, 10 and 12 h after the dosing for about 0.3 mL each time. Each sample was centrifuged at 1650 × *g* for 10 min. The plasma obtained after the dosing was mixed, and all plasma was stored at −20 °C until analyzed.

#### 2.3.2. Urine and feces sample collection

Three of rats had been housed individually in metabolic cages for a week and allowed access to water and food freely. The urine and feces samples were both collected for 24 h before and after the dosing, respectively. The urine sample was centrifuged at 1650 × *g*

**Table 2**
All the metabolites detected after an oral administration of BBR (25 mg kg$^{-1}$) to rats.

| Phase[a] | No. | $t_R$ (min) | [M−H]$^-$ | | | | Source[b] | Compound presumed |
|---|---|---|---|---|---|---|---|---|
| | | | Calculated | Detected | Error (ppm) | Sigma | | |
| P | 0 | 25.5 | 420.9080 | 420.9059 | −5.7 | 0.009 | p, u, f, b | $C_{17}H_{12}Br_2O_3$ |
| I | 1[c] | 7.1 | 470.9084 | 470.9084 | 0.0 | 0.029 | p, u | $C_{17}H_{14}Br_2O_6$ |
| I | 2[c] | 11.2 | 454.9135 | 454.9100 | −8.2 | 0.017 | p, f, u | $C_{17}H_{14}Br_2O_5$ |
| I | 3 | 11.7 | 498.8940 | 498.8894 | −9.2 | 0.023 | u | $C_{18}H_{14}Br_2O_7$ |
| I | 4 | 12.9 | 452.8979 | 452.8946 | −5.5 | 0.013 | p, u, f, b | $C_{17}H_{12}Br_2O_5$ |
| I | 5 | 16.7 | 436.9030 | 436.9025 | −3.4 | 0.029 | p, u, f, b | $C_{17}H_{12}Br_2O_4$ |
| I | 6 | 18.1 | 436.9030 | 436.9014 | −2.4 | 0.010 | p, u, f, b | $C_{17}H_{12}Br_2O_4$ |
| I | 7 | 18.9 | 436.9030 | 436.9036 | 1.9 | 0.041 | p, u, f, b | $C_{17}H_{12}Br_2O_4$ |
| II | 8 | 6.5 | 628.9300 | 628.9279 | −3.2 | 0.008 | b | $C_{23}H_{20}Br_2O_{11}$ |
| II | 9 | 6.9 | 628.9300 | 628.9277 | −3.0 | 0.008 | b | $C_{23}H_{20}Br_2O_{11}$ |
| II | 10 | 8.3 | 628.9300 | 628.9292 | −0.2 | 0.009 | u, b | $C_{23}H_{20}Br_2O_{11}$ |
| II | 11 | 10.2 | 612.9350 | 612.9395 | 7.1 | 0.011 | b | $C_{23}H_{20}Br_2O_{10}$ |
| II | 12 | 11.0 | 612.9350 | 612.9389 | 5.3 | 0.019 | b | $C_{23}H_{20}Br_2O_{10}$ |
| II | 13 | 12.0 | 612.9350 | 612.9374 | 3.4 | 0.016 | u, b | $C_{23}H_{20}Br_2O_{10}$ |
| II | 14 | 14.5 | 514.8441 | 514.8492 | 9.9 | 0.037 | u | $C_{17}H_{10}Br_2O_7S$ |
| II | 15 | 17.5 | 498.8492 | 498.8527 | 7.0 | 0.046 | p, f, u | $C_{17}H_{10}Br_2O_6S$ |
| II | 16 | 23.5 | 532.8547 | 532.8593 | 8.6 | 0.018 | u, b | $C_{17}H_{12}Br_2O_8S$ |
| II | 17 | 26.5 | 516.8598 | 516.8569 | −5.7 | 0.018 | u | $C_{17}H_{12}Br_2O_7S$ |

[a] Phase: p, parent drug; I, phase I metabolite; II, phase II metabolite.
[b] Source: p, plasma; u, urine; f, feces; b, bile.
[c] The reduced metabolites.