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BMCL26; Anti-parasitic; LC-MS/MS; Protein precipitation; Rat plasma

%CV: Coe cient of Variation; IS: Internal Standard; LLOQ: Lower Limit Of Quanti cation; MRM: Multiple Reaction Monitoring; AMP: Aminoprophos-Methyl; JCC76: N-(3-(2,5-dimethylbenzyloxy)-4-(methylmethylsulfonamido)phenyl)cyclohexanecaboxamide; %RE: Relative Error; RME: Relative Matrix E ect; QC: Quality Control; SD: Standard Deviation

Human African trypanosomiasis, also known as sleeping sickness, is a vector-borne parasitic disease and a serious health threat to a large number of people living in sub-Saharan Africa, where health systems are challenged at best [1-3]. *Trypanosoma brucei gambiense* (*T. b. gambiense*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense)* are the etiological parasites that cause sleeping sickness in humans. ese parasites live and grow extracellularly in the blood and tissue uids of humans or cattle and are transmitted among hosts by tsetse ies (*Glossina* spp.). Without e ective treatment, the disease can lead to coma and ultimately death. If the patients do not receive treatment in a timely manner, the neurological damage caused by these parasites is irreversible even a er treatment [3,4]. Current drugs used to treat human trypanosomiasis include Suramin, Pentamidine, Melarsoprol and E ornithine [5], however, these drugs do not e ectively treat the disease, maintaining an urgent need for new, more e ective and less expensive drugs for the treatment of human African trypanosomiasis [4-6].

Tubulin is a very attractive target in the eld of anti-cancer drug discovery, and several successful tubulin binders are used clinically as rst-line chemotherapeutic agents [7]. Tubulin also plays an essential role during trypanosome cell division. e fast, population-doubling rate of trypanosomes makes them highly dependent on tubulin polymerization/depolymerization [8]. More importantly, tubulin is critical for trypanosome locomotion, which is essential for trypanosomes survival. Tubulin inhibitors not only block *T. brucei* cell division but also a ect the locomotive functions of agellum and lead to cell death [9]. Some microtubule-disrupting herbicides, such as phosphoric thioamide herbicide Amiprophos-Methyl (APM) and dinitroaniline herbicides, exhibit activity against protozoan parasites by targeting tubulin [10-14]. Research works have optimized these compounds, generating more potent and selective tubulin inhibitors for *T. brucei* [10]. Webovertz's group successfully developed several drug candidates that show promising anti-parasite activity and selectivity *in vitro.* However, these compounds did not show substantial potency *in* 39on T. brity

Methanol (HPLC grade) and acetonitrile were from Pharmco-Apper (Philadelphia, Pennsylvania, USA). Formic acid and ammonium acetate (analytical grade) were purchased from Sigma Aldrich Chemical Company (Allentown, Pennsylvania, USA). Deionized water was obtained using a Barnstead Nano pure water puri cation system with a Nanopure Diamond Pack Organic free DI cartridge from ermo Scienti c (Waltham, Massachusetts, USA). Six individual lots of rat plasma (Sprague-Dawley rat plasmas K2) were obtained from Innovative Research (Novi, Michigan, USA) (Figure 1).

[illegible]

[illegible] A set of BMCL26 working solutions containing 10, 20, 50, 150, 400, 1000 and 2000 ng/mL were prepared by serial dilution using methanol and 1 mg/mL stock solution. e 150 ng/mL working solution of JCC76 (IS) was diluted from a stock solution of 1 mg/mL in methanol. Stock solutions and working solutions were stored at -20°C and 4°C.

[illegible]

Calibration plasma samples were prepared by spiking 10 µl of corresponding BMCL26 working solutions in 200 µl of rat plasma (mixture of 6 lots) with drug concentrations of 0.5, 1.0, 2.5, 5, 12.5, 25, 50, and 100 ng/mL. QC samples at three concentrations, 1.25 (low), 10 (mid) and 80 (high) ng/mL, were prepared by adding 10 µL of the appropriate BMCL26 working solution and 200 µL of drug-free plasma. Calibration and QC samples were frozen at -20°C overnight and then treated using the following sample preparation procedure before LC-MS/MS analysis.

[illegible]

QC samples and blanks were removed from the -20°C freezer and thawed to room temperature. Ten µl of JCC76 working solution were spiked into each 200 µL aliquot of plasma calibrators/QCs/blanks, excepting the double blank, into which 10 µL of acetonitrile was added. e solutions were then vortexed immediately for 30 sec, a er which each sample was deproteinized by adding 800 µl of 0.1% formic acid in acetonitrile, sonicating for 15 minutes, and centrifuging at 13,000 × g for 15 minutes. e supernatants were then transferred into auto sampler vials for LC-MS/MS analysis.

[illegible]

LC-MS/MS analysis was performed using a 5500 Q-TRAP triple quadrupole, tandem mass spectrometer (AB Sciex, Toronto, Canada) coupled with an Electrospray Ionizer (ESI) operated in negative ion mode (Framingham, Massachusetts, USA) and interfaced with High Performance Liquid Chromatography (HPLC, Shimadzu, Columbia, Maryland, USA) system that uses auto-sampling and online vacuum degassing. All data acquisition and processing were conducted using Analyst so ware, version 1.5.2 (AB Sciex).

Analytical separation of BMCL26 was achieved using a Luna C8 (2) HPLC column (50 × 2.0 mm, 5 micron) with a C8 security-guard cartridge from Phenomenex (Torrance, California, USA). Mobile phase A contained 50 µM ammonium acetate in 2% Methanol, and mobile phase B contained 50 µM ammonium acetate in 90% Methanol. Sample aliquots of 5 µl were injected onto the column and eluted via the following gradient flow: 0-0.6 min, 70% B, 1.6 min, 90% B, 7.5 min, and stop (Table 1). The column was equilibrated for 0.5 min before each run. Negative electrospray ionization (ESI) mode was selected, and the MRM (multiple reaction monitoring) function was used for quantification, with the transitions set at m/z 573.3 → 493.2 for BMCL26 and m/z 443.2 → 79.1 for JCC76 (IS) (Figure 2), respectively. The following ion-source-dependent parameters were used: nebulization gas (30), heating gas (30), curtain gas (35), ion spray voltage (-4330 ev) and temperature (500°C). Compound-dependent parameters were manually optimized as follows: declustering potential, -40; entrance potential, -10; collision energy and cell exit potential for both analyte and internal standard, -30, -100, -13, and -9.

of 0.5-100 ng/mL. Linearity results showed a quadratic t for BMCL26 using an eight-point calibration curve (0.5, 1, 2.5, 5, 12.5, 25, 50, and 100 ng/mL) with JCC76 (7.5 ng/mL) as the internal standard in the plasma samples. Excellent linearity was obtained with a correlation coe cient (r^2) of 0.9993. e linear regression equation was y=0.073x-0.0085. is method exhibited high selectivity and displayed no interfering peaks in six di erent blank plasma samples from di erent sources. Using the calibration curve, the LLOQ of the method was determined to be 0.5 ng/mL. e accuracy and precision were determined for each lot of plasma at the LLOQ. e data were summarized in Table 2.

[illegible]

Intra- and inter-assay accuracies (%RE) and precisions (%CV) were evaluated by analyzing ve replicates of low, medium, and high QC standards. As summarized in Table 3, the assay's intra- and inter-day relative errors were 0.62 and 11.36%, respectively, and the assay's intra- and inter-day precisions were 0.84-3.47%, respectively. ese

mL) QC samples to those of freshly prepared QC solutions (containing the same concentrations), expressed in terms of recovery. As shown in Table 6, the receives of LQC and HQC samples were 103.46-103.20%, 98.13-105.20%, and 101.86-107.54% for bench top conditions, a er 3 freeze-thaw cycles and post extraction at room temperature for 10 hours, respectively. e stabilities of working solutions of BMCL26 and internal standard (JCC76), stored at 4°C for at least 6 months, were determined to be 99.60-105.00% and 115.60% for the two QC standards tested (1.25 and 80 ng/mL), to which 7.5 ng/mL IS was added (using the stored stock solution). ese stability results showed no signi cant deviations in BMCL26 quanti cation under the experimental conditions used.