2.2.2.4 Method of extraction

An appropriate number of Eppendorf tubes were placed in a rack and labeled properly, 100 µl aliquots of each test sample (*In vivo* rat samples, *in situ* SPIP, everted gut samples) was pipetted into the appropriate labeled tube followed by the addition of 150 µl IS (5 µg/ml of Sildenafil). Finally, each sample was vortexed vigorously for 1 min then centrifuged at 14000 rpm for 15 minutes. The procedure described was applied for subject samples, calibrator and quality control samples.

2.2.2.5 Method development

By referring to previous studies, PRN was detected in plasma using reverse phase HPLC method. PRN wavelength was determined after several trials on different wavelength of 250, 280 and 214 nm resulting in the best detection for 214 nm wavelength. IS selection depends on chemical similarity of this IS to PRN, comparable retention times and similarly in the derivatization procedures of both drugs. Good separation between PRN HCl and sildenafil was obtained after the examination of different mobile phases and columns. Among serum extraction methods, protein precipitation method with acetonitrile was found to be optimal in our method for the extraction of serum and Krebs buffer. Mobile phase composition was selected after several trials to obtain symmetric shapes of analyte and good resolution with short run time (7 min).