A sensitive, simple and selective high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method was developed to determine the norcantharidin concentrations in human serum and tissues with cyclophosphamide as internal standard (I.S.), and its possible metabolites were qualitated. Norcantharidin (NCTD) and I.S. in serum and in tissues were extracted with acetone, separated on a C18 reversed-phase column, gradient eluted with mobile phase of acetonitrile/water containing 2 mM ammonium acetate and 0.1% formic acid (pH3), ionized by positive ion pneumatically assisted electrospray and detected in the multi reaction monitoring (MRM) mode using precursor \( \rightarrow \) product ions of \( m/z \) 169.3 \( \rightarrow \) 123.1 for NCTD and 261.2 \( \rightarrow \) 140.2 for I.S., respectively. The linear range of the calibration curve for NCTD was 2.5-50 ng•mL\(^{-1}\), with the lowest limit of quantification (LOQ) of 2.5 ng•mL\(^{-1}\), and the intra/inter-day RSD were less than 10%. The established HPLC-MS/MS method was suitable to meet the requirement of determination of low NCTD concentration in human serum after therapeutic oral doses, and has been successfully used to the pharmacokinetic studies in healthy Chinese volunteers. Two possible metabolites were verified, and the tissues distribution studies shown that NCTD was aggregated in some organs like ovary, not the labeled indication, kidney, may define the cause of NCTD’s kidney toxic effects, but not liver, the labeled indication.

**Keywords:** norcantharidin, HPLC-MS/MS, pharmacokinetic, cyclophosphamide