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Scientific paper

Determination of Copper at Extended Dose Levels of Copper (II)-acetylsalicylate and Pharmacokinetics Applications

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Abstract

Rheumatoid arthritis has long been treated with acetylsalicylic acid, despite many side effects, including gastric ulcers. These side effects can be curtailed by preparing the to metal complexes of acetylsalicylic acid acid, such as copper (II)-acetylsalicylate (CAS). Present study evaluates the pharmacokinetics parameters of CAS and the level of copper at extended dose levels using rabbit model. The concentrations of CAS and copper in plasma samples were determined by validated HPLC and atomic absorption spectroscopic (AAS) methods, respectively. Three doses, 1–3 mg kg^{-1} were orally administered to six rabbits with two wash out periods. The blood samples were collected at different time intervals for 24 hours. The peak drug concentration (C_{max}) for these doses at a time to peak drug concentration (t_{max}) 0.5 h was determined to be 0.38, 0.76 and 1.14 μg mL^{-1}. The half-life of drug ($t_{1/2}$) was 8.67, 8.73 and 8.81 h, which are perfect results for once a day dosing. The values of volume of distribution (V_d) and clearance (Cl) for CAS were 829, 833 and 837 L kg^{-1} and 66.30, 66.74 and 66.95 L h^{-1}, respectively. The AAS results showed that copper levels in rabbit blood plasma were increased with increasing the dosage of CAS, but still remains under the safer limit, which was twofold higher than the reported safe limit.

Keywords: Copper (II)-acetylsalicylate, Copper level, Atomic Absorption Spectroscopic Method, Pharmacokinetics, HPLC-UV

1. Introduction

Acetylsalicylic acid (ASA) is commonly used for the treatment of rheumatoid arthritis since the last century. However, prolonged use of this medication is associated with many side effects. Peptic ulcers are among the major side effects ascribed to ASA.[1,2] One approach to diminish these serious gastric side effects is the development of polymer based prodrugs of salicylic acid and ASA.[3, 4] However, the synthesis of copper (II)-acetylsalicylate (CAS) by Sorenson (1976) not only subsided these side effects, but it was also found to possess antiulcer potential and with enhanced activity.[5–8] Many other copper complexes have also been found to possess greater anti-inflammatory(AI) activity than their parent compounds or ligands, therefore copper complexes have attracted a great attention as anti-arthritics and AI drugs since the last two to four decades.[9–11] The only possible hindrance left behind for the safer use of CAS as anti-rheumatic drug is the rationalization between concentration of copper metal and its levels in the body. Recently, we have reported that CAS has improved pharmacokinetics compared with ASA after administration of a single oral dose in the human volunteers.[12] However, plasma copper levels, while using moderate to higher dose levels of CAS were not carried out and need to be determined.

In the present study, pharmacokinetics parameters of CAS with enhanced dose along with the monitoring of plasma copper level is presented for the first time by validated HPLC/UV and atomic absorption spectrophotometric (AAS) methods.

2. Materials and Methods

2. 1. Participants and Study Design

Six white male albino rabbits, weighing 1.4–1.8 kg were used from the laboratory animal house, University of Sargodha, Sargodha, Pakistan. All the rabbits were healthy and never used previously for any type of studies. The animals were kept in separate cages under a 12 h light/dark cycle and were given free access to pelleted feed concentrate and water. A crossover design was used in three phases (2 × 2 × 2), with two washout periods of 15 days each. The rabbits were kept on fast for at least 10 h (overnight). Rabbits were prevented from taking water 1 h before the drug administration. A pre-dose blood sample was taken from all the rabbits, which was termed and used as blank. In the first phase, a single dose of 1.0 mg kg^{-1} of CAS with water was administered to six rabbits. After the blood sampling, the rabbits were fed with natural food and given a washout period of 15 days. The given procedure was repeated for second and third blood sampling at dosage of 2.0 mg kg^{-1} and 3.0 mg $kg^{-1}$of CAS, respectively. The study protocol was approved by the Institutional Animal Ethics Committee (IEC), Faculty of Pharmacy, University of Sargodha (Approval No.31-C12 IEC UOS). All the experiments performed complied with the rulings of National Research Council.[13]

2. 2. Specimen Collection

Blood samples (3–5 mL) were taken from jugular vein of each rabbit using heparin (Leo, Denmark) added disposable syringes (*Injekt*) under aseptic conditions. The blood samples were transferred to blood collection tubes (BD Vacutainer®) and labeled consequently with great care. Post-dose blood samples were collected after 0.15, 0.3, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12 and 24 h. The blood samples were centrifuged at 4000 x *g* for 5 min and supernatant layer (plasma) of each sample was separated carefully and freezed.

2. 3. Specimen Analysis

The test plasma sample (1.0mL) was thawed quickly under cold water and then promptly but briefly vortex mixed and processed further for protein precipitation according to a reported method by M. S. Iqbal *et al.* (2008)[12]. The plasma samples were analyzed by an already reported HPLC/UV method[12] and concentrations of CAS were determined. All the chemicals used for HPLC analyses were of analytical reagent grade and obtained from Merck, Germany. Standard samples of CAS were prepared according to reported method[12]. The HPLC/UV system consisted of: LC-10 ATVP pump, UV-Vis detector SPD-10A VP, and SCL-10A VP system controller all from Shimadzu, Japan. The column used was Shim-pack ODS (5 μm, 4.6 mm i.d.× 250 mm). The methanol and acetic acid were used as mobile phase in a ratio of 20:01. The flow rate, detection wavelength and injection volume used were 1.0 mL min^{-1}, 294 nm and 20μL, respectively.

2. 4. Pharmacokinetic Analysis

Concentration-time curves were plotted and the pharmacokinetic parameters i.e., area under curve from time zero to time t (AUC_{0-t}) and area under curve from time zero to time infinity ($AUC_{0-\infty}$) were calculated by the following formula;

$$AUC_{0-\infty} = AUC_{last} + C_t/ke$$

Where, $AUC_{0-\infty}$ is the area under curve from time zero to time infinity, C_t, concentration at a particular time and *ke,* absorption constant. Other parameters include, $t_{½}$ = 0.693/*k*e, half-life of drug; C_{max}, peak drug concentration; t_{max}, time to peak drug concentration.

Area under the concentration-time curve was determined by the linear trapezoidal method. The terminal rate constant was resolved by regression analysis of at least three data points in the terminal phase. One way analysis of variance (ANOVA) was used to compare variations in the results obtained at extended dose levels.

2. 5. Copper Analysis

The copper level in plasma was measured by an AAS method using graphite furnace.[14] The instrument used was an AA 6300 (Shimadzu, Japan) operational with copper hollow cathode lamp and GFA-EX7i graphite furnace. A 20 μL pipette was used for sample injection and the instrument operating conditions were as follows: wavelength: 324.7 nm, lamp current: 8mA, spectral band width: 0.7 nm, drying time: 30 s, drying temperature: 120 °C, ashing time: 30 s, ashing temperature: 800 °C, atomization time: 10 s, atomization temperature: 2500 °C and argon flow rate: 1.5 L min^{-1} at 40 psi.

Stock solution of copper: A 1000 μg mL^{-1} of copper stock solution was prepared from copper acetate diluted with double-distilled water. Working standard solution of copper: 1.0 mL of stock solution of copper (1000 μg mL^{-1}) was transferred into a 100 mL calibrated flask and made up to the mark with double-distilled water to provide a working standard solution of copper having a concentration of 10 μg mL^{-1}. 2, 4, 6,…, 18 and 20 mL of 10 μg mL^{-1} copper solutions were added separately to the 100 mL calibrated flasks and made the volume up to the mark with double-distilled water. Resulting solutions were of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 μg mL^{-1} concentration, respectively. These solutions were used as working standard solutions of copper.

Preparation of sample solution: 1.0 mL of 10^{-2} M nitric acid was added to 1.0 mL of blank plasma, heated, cooled and then transferred to auto sampler of graphite atomic absorption spectrometer. Other plasma sample

taken at time intervals 0.15, 0.3, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12 and 24 h of three doses (1.0, 2.0 and 3.0 mg kg^{-1} body weight of rabbit) were prepared by the procedure used for blank plasma.

3. Results and Discussion

3. 1. Pharmacokinetic Evaluation

The pharmacokinetic analysis was completed with the help of a model independent method. The C_{max} as well as the t_{max} of CAS were measured by assessment of the individual drug plasma concentration-time profiles. The elimination rate constant (k_e) was attained from least-square fitted terminal log-linear portion of the plasma concentration-time profile. The $t_{1/2}$ was considered equal to $0.693/k_e$. AUC_{0-t} was evaluated by linear trapezoidal rule. $AUC_{0-\infty}$ was considered equal to $AUC_{0-t} + C_t/k_e$ whereas, C_t is the last measurable concentration. In the pharmacokinetics analysis, AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were regarded as primary variables and converted to logarithmic terms.

The pharmacokinetic parameters for CAS after oral administration of 1.0, 2.0 and 3.0 mg kg^{-1} are given in Table 1. The C_{max} in case of 1.0 mg kg^{-1} dose was determined 0.38 μg mL^{-1} at a t_{max} 0.5 h and the $t_{1/2}$ was 8.67 h. However, volume of distribution (V_d) and clearance (Cl) values for CAS were 829 L kg^{-1} and 66.30 L h^{-1}, respectively. In case of 2 mg kg^{-1} dose, the C_{max} was determined 0.76 μg mL^{-1} at a t_{max} 0.5 h and the $t_{1/2}$ was found 8.73 h. Nevertheless, V_d and Cl values for CAS were 833 L kg^{-1} and 66.74 L h^{-1}, respectively. Whereas, in 3 mg kg^{-1} dose the C_{max} was calculated to be 1.14 μg mL^{-1} and the $t_{1/2}$ was 8.81 h. The V_d and Cl values for CAS were obtained to be 837 L kg^{-1} and 66.95 L h^{-1}, respectively. In all three studies, the value of $t_{1/2}$ is greater than 8 h, which is ideal for once a day dosing. The massive V_d might be as a result of absorption by a particular tissue or membrane, as very lipophilic compounds are recognized to distribute into lipids in cell membranes and fat stores; these competently form slow discharge depots of the drug and extend the plasma levels.[15] The comparatively large clearance may direct to low exposure and less plasma average concentrations throughout chronic dosing.

In pharmacokinetic evaluation studies, it was observed that with the increase in dose, there is a change in the C_{max} value for each dose, whereas the values for other parameters do not show any significant difference from one another. It clearly indicates that the absorption and elimination behavior of the drug is same in all cases and it was independent of dose administered except the value of C_{max}. The pharmacokinetic results obtained in this study are quite consistent and determine the safety of CAS at extended dose levels.

3. 2. Copper Level Determination

The plasma copper levels were measured by AAS at 0.0, 0.15, 0.3, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 8.0, 12 and 24 h after oral administration of three doses of CAS 1.0, 2.0 and 3.0 mg kg^{-1} body weight of six rabbits (A, B, C, D, E and F) with two wash periods of 15 days. The average concentration of copper in plasma of six rabbits at different time intervals for extended doses (1, 2 and 3 mg kg^{-1} body weight of rabbits) is recorded and the trend is shown in Figure 1.

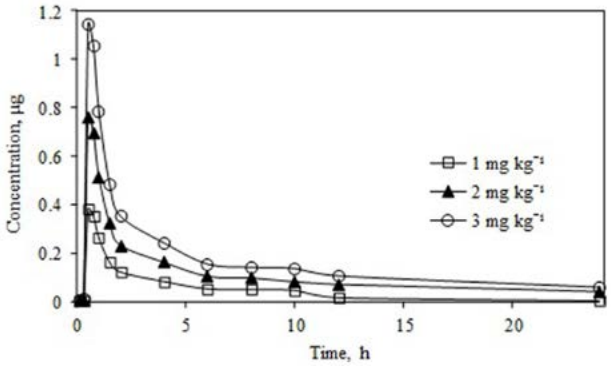

Figure 1. Overlay plot of copper concentrations (μg) vs. time (h) at different dose levels.

The trend in the Figure 1 shows that the concentration of copper in plasma increases with an increase in the concentration of CAS. There is an increase in the level of copper up to 0.5 h and later on the level of copper drops down to acceptable limits. Moreover, two way ANOVA (Tukey's multiple comparison test) revealed that extended dose of 3 mg kg^{-1} showed some variations in terms of significance as compared to the dose of 1 mg/kg levels up to 1 h. Afterwards, no significant variation in plasma concentration of copper was documented in the three

Table 1. Pharmacokinetic data after oral dose of 1.0, 2.0 and 3.0 mg of CAS per kg body weight.

	Pharmacokinetic parameters						
Dose (mg kg^{-1})	**t_{max} (h)**	**C_{max} (μg mL^{-1})**	**$t_{1/2}$ (h)**	**AUC_{0-t} (h μg L^{-1})**	**$AUC_{0-\infty}$ (h μg L^{-1})**	**V_d (L kg^{-1})**	**Cl (L h^{-1})**
1.0	0.5	0.38	8.67	0.71	0.91	829	66.30
2.0	0.5	0.76	8.73	0.74	0.95	833	66. 74
3.0	0.5	1.14	8.81	0.75	0.97	827	66.45

Table 2. Two way ANOVA chart; mean plasma concentration vs. time at extended dose levels.

Dosage	**0.15 h**			**0.3 h**			**0.5 h**		
	Mean	**±**	**Standard error (S.E)**	**Mean**	**±**	**S.E**	**Mean**	**±**	**S.E**
1mg kg^{-1}	0.003	±	0.0006	0.003	±	0.001	0.38	±	0.02
2 mg kg^{-1}	0.004	±	0.0006	0.005	±	0.0008	0.76	±	0.05
3 mg kg^{-1}	0.005	±	0.0008	0.093	±	0.026	1.14	±	0.06***
Dosage	0.75 h			1 h			1.5 h		
	Mean	±	S.E	Mean	±	S.E	Mean	±	S.E
1 mg kg^{-1}	0.35	±	0.03	0.263	±	0.017	0.16	±	0.02
2 mg kg^{-1}	0.69	±	0.079	0.513	±	0.076	0.32	±	0.07
3 mg kg^{-1}	1.05	±	0.03***	0.78	±	0.048*	0.48	±	0.03
Dosage	2 h			4 h			6 h		
	Mean	±	S.E	Mean	±	S.E	Mean	±	S.E
1 mg kg^{-1}	0.12	±	0.02	0.08	±	0.012	0.051	±	0.008
2 mg kg^{-1}	0.23	±	0.07	0.16	±	0.031	0.103	±	0.005
3 mg kg^{-1}	0.35	±	0.035	0.24	±	0.012	0.153	±	0.007
Dosage	8 h			10 h			12 h		
	Mean	±	S.E	Mean	±	S.E	Mean	±	S.E
1 mg kg^{-1}	0.05	±	0.004	0.045	±	0.003	0.016	±	0.004
2 mg kg^{-1}	0.10	±	0.008	0.083	±	0.02	0.07	±	0.014
3 mg kg^{-1}	0.14	±	0.016	0.135	±	0.006	0.105	±	0.006
Dosage	24 h								
	Mean	±	S.E						
1 mg kg^{-1}	0.002	±	0.0004						
2 mg kg^{-1}	0.042	±	0.012						
3 mg kg^{-1}	0.058	±	0.007						

* show statistically significant variation in plasma concentration of copper at different time intervals when comparing between group 1&3 at significance level of $\alpha = 0.05$

groups. Nevertheless, the plasma concentration of copper achieved from this dose of 3 mg kg^{-1} remains within safe therapeutic limits.

4. Conclusion

The animal model (rabbits) were used to evaluate the pharmacokinetics parameters of CAS and copper level at extended doses of CAS. Validated HPLC and AAS methods were employed to determine the plasma concentrations of CAS and copper, respectively. Results of pharmacokinetic studies have revealed that copper level in plasma increases under safer limits up to 2 mg kg^{-1} dose level that is equivalent to 120 mg human dose. It is two-fold higher safe limit than the reported of dose 60 mg, i.e., useful for rheumatoid arthritis and therefore a higher dose of 2 mg kg^{-1} can be used safely. It is also worth mentioning here that the dose 3 mg kg^{-1}, which is equivalent to 180 mg can also be used. Although at time interval 0.5 h, copper levels increase (1.9 mg kg^{-1}) little up to the acceptable level of copper in plasma (0.5–1.7 mg kg^{-1}), however within about 15 min the plasma maintains the copper levels under the acceptable limits. Therefore, present work indicates that CAS is a safe prodrug of ASA even at three fold extended dose levels than a normal dose i.e. 60 mg suggested for humans.[12] However, the toxicity due to high dose may be determined by analyzing the accumulation of copper metal in different tissues of the body.

Conflict of Interest Statement

The authors confirm that this article content has no conflict of interest.

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Povzetek

Revmatoidni artritis so dolgo zdravili z acetilsalicilno kislino, kljub njenim številnim neželenim učinkom, ki vključujejo želodčne razjede. Te neželene učinke je mogoče zmanjšati s pripravo kovinskih kompleksov acetilsalicilne kisline, kot je bakrov (II)-acetilsalicilat (CAS). Pričujoča študija ocenjuje farmakokinetične parametre CAS in raven bakra pri povečanih odmerkih na modelu kunca. Koncentracije CAS in bakra v plazemskih vzorcih so določili z validirano HPLC metodo in atomsko absorpcijsko spektroskopijo (AAS). Šestim kuncem so peroralno aplicirali tri odmerke, 1–3 mg kg^{-1}, z dvema obdobjema izpiranja. Vzorce krvi so zbirali v različnih časovnih intervalih znotraj 24 ur. Največja koncentracija učinkovine (C_{max}) za te odmerke pri času do največje koncentracije učinkovine (t_{max}) 0,5 h je znašala 0,38; 0,76 in 1,14 μg mL^{-1}. Razpolovna doba učinkovine ($t_{1/2}$) je znašala 8,67; 8,73 in 8,81 h, kar so ustrezni rezultati za odmerjanje enkrat na dan. Vrednosti volumna porazdelitve in očistka za CAS so po vrsti znašale 829, 833 in 837 L kg^{-1} ter 66,30, 66,74 in 66,95 L h^{-1}. Rezultati AAS so pokazali, da so se vrednosti bakra v krvni plazmi kuncev povečale z večanjem odmerka CAS, vendar so še vedno ostale pod varnejšo mejo, ki je bila dvakrat višja od priporočene varne meje.