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Determination of a prodrug of tranexamic acid in whole blood by reversed-phase liquid chromatography after pre-column derivatization with fluorescamine*

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Abstract: A sensitive high-performance liquid chromatographic (HPLC) method for a prodrug of tranexamic acid (KABI 2161) in whole blood is described. Since KABI 2161 is rapidly hydrolysed in whole blood the samples are collected directly into the extraction tubes and extracted immediately. After pre-column derivatization with fluorescamine the derivatives are analysed by reversed-phase liquid chromatography on a C_8-Nucleosil column using an eluent mixture of phosphate buffer and acetonitrile (pH 3). The eluent is monitored by a fluorescence detector. Determinations as low as 10 ng ml^{-1} of KABI 2161 in whole blood can be made when 0.5 ml blood is analysed. The precision of the method is 4.1% (RSD) at the 300 ng ml^{-1} level and 6.9% (RSD) at the 50 ng ml^{-1} level.

Keywords: *Reversed-phase liquid chromatography; tranexamic acid prodrug; whole blood; pre-column derivatization; fluorescence.*

Introduction

The development of prodrugs to increase drug absorption is a field of growing activity and has been applied to drugs of different origin [1, 2]. Thus, in order to increase the absorption after oral administration of the antifibrinolytic drug tranexamic acid (trans-4-aminomethyl-cyclohexanecarboxylic acid), a prodrug of ester type (acylal) was developed [3]. To demonstrate its rapid degradation *in vivo* a sensitive analytical method for the intact prodrug in the presence of tranexamic acid was required. This paper presents a method for the quantitative determination of KABI 2161 in whole blood used in toxicological and pharmacological experiments. Since KABI 2161 is an ester which is rapidly hydrolysed in whole blood, special precautions in the sample handling and isolation steps had to be taken. A rapid extraction at physiological pH was desirable. This was achieved by ion-pair extraction using bromothymolblue as counter-ion [4]. The whole blood samples were collected directly into the extraction tube and immediately

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extracted with bromothymolblue into methylene chloride and then re-extracted to an aqueous phase containing tetrabutylammonium hydrogensulphate. The extracted prodrug was derivatized with fluorescamine, a well known reagent for primary amines [5, 6]. The underivatized prodrug exhibits only weak UV-absorption at 210 nm. Stability problems during sample handling are discussed as well as the optimization of the derivatization step.

Experimental

Apparatus

The pump was a Constametric IIG (Laboratory Data Control, Riviera Beach, Florida, USA) operated at a flow rate of 1.0 ml min^{-1}. The reversed-phase column (150 × 4.6 mm) contained 5-μm C_8-Nucleosil particles (Macherey-Nagel & Co., Düren, FRG) and the guard column (40 × 4.6 mm) connected to it was a Spheri-5 RP8, MPLC (Brownlee Labs Inc., Santa Clara, USA). A Wisp 710 B automatic injector (Waters Associated Inc., Milford, MA, USA) with the injection volume set to 100–200 μl was used. The fluorescence was monitored by a Schoeffel FS-970 fluorescence detector (Kratos, Schoeffel Instruments, FRG) with the excitation wavelength set to 280 nm and with an emission filter of 470 nm. The sensitivity was set to 0.1–0.2 μA when samples with a concentration of prodrug between 10 and 100 ng ml^{-1} were analysed. A recorder (Servogor, Wien, Austria) with a 10 mV full scale deflection and a chart speed of 5 mm min^{-1} was used. In some of the extraction studies the underivatized prodrug was monitored by a Spectromonitor III variable detector (Laboratory Data Control, Riviera Beach, Florida, USA) operated at 210 nm. Fluorescence spectra were recorded by an Aminco Bowman Spectrophotofluorometer (American Instrument Company, Silver Spring, Maryland, USA) with the excitation wavelength set to 420 nm and the emission wavelength set to 485 nm. A Sorvall GLC-2B centrifuge (Du Pont Instruments, CT, USA) operated at 1650 ***g*** was used. All glassware used in the method was carefully cleaned with water, Decon solution (Decon Laboratories, Hove, UK) and ethanol and stored isolated from other laboratory glassware.

Chemicals and reagents

KABI 2161 and the internal standard OAF 71901 were synthesized at KabiVitrum as their hydrochlorides. Figure 1 shows their chemical structures as well as their reaction with fluorescamine. Acetonitrile (Rathburn Chemicals Ltd, Walkerburn, Scotland) was of HPLC Grade S; methylene chloride (Fisher, New Jersey, USA) was of analytical quality, and shaken with water before use. Aqueous buffer solutions were prepared using appropriate amounts of sodium dihydrogenphosphate or disodium hydrogenphosphate and phosphoric acid. Bromothymolblue (BTB), 0.01 M, pH 8.0–8.5, was prepared freshly every month by dissolving 6.46 g bromothymolblue sodium salt (Fluka, Buchs, Switzerland) in 8 ml of 1 M sodium hydroxide and diluting to 1000 ml with water. Tetrabutylammonium hydrogensulphate (TBAHS), 0.05 M was prepared by dissolving 8.5 g of tetrabutylammonium hydrogensulphate (Fluka, Buchs, Switzerland) in 500 ml of phosphate buffer, pH 3, $\mu = 0.1$. Fluorescamine 0.4%, w/v, in acetonitrile was prepared by dissolving 100 mg of fluorescamine (Hoffman–La Roche, Basle, Switzerland) in 25 ml of acetonitrile. Stock solutions (80 μg ml^{-1}) were prepared by dissolving 20 mg of KABI 2161 in 250 ml of phosphate buffer pH 4, $\mu = 0.1$ and by dissolving 20 mg of the internal standard in 250 ml of water. The internal standard solution (4.0 ml) was further diluted

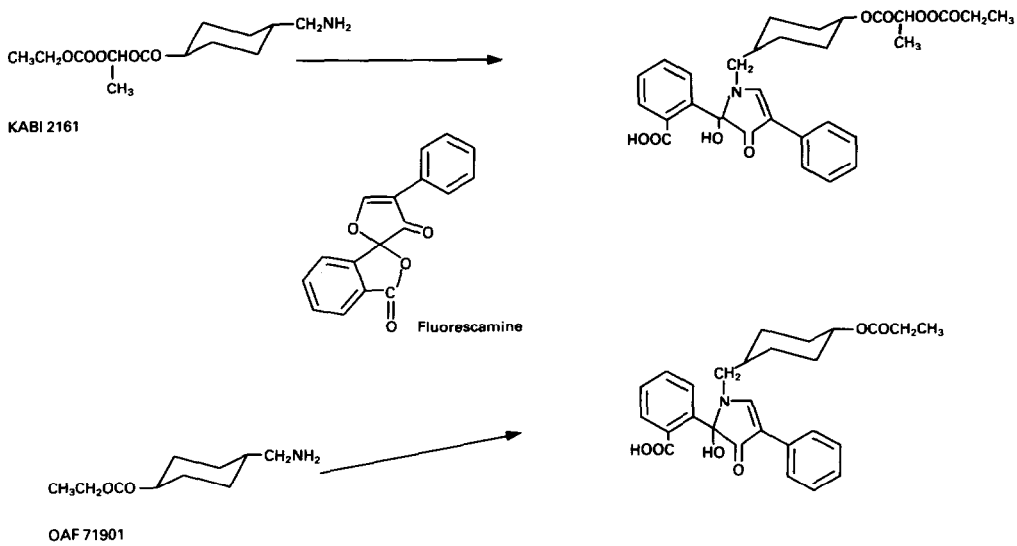

Figure 1
Structures of KABI 2161 and the internal standard OAF 71901 and their reaction with fluorescamine.

to 50.0 ml with phosphate buffer pH 4, $\mu = 0.1$ to obtain 6.4 μg ml^{-1}. The solutions could be stored at 20°C for about one month. Internal standard solution (32 ng ml^{-1}) was prepared freshly every day by diluting 1 ml of the stock solution to 200 ml with 0.01 M BTB. When samples containing >200 ng ml^{-1} of KABI 2161 were analysed the concentration of this solution was doubled (i.e. 64 ng ml^{-1}). Standard solutions were prepared freshly every day by diluting 2 ml of the stock solution (80 μg ml^{-1}) to 100 ml with water (1.6 μg ml^{-1}). This solution was diluted further with the internal standard solution (32 ng ml^{-1}) in BTB, to give concentrations of 100, 50, 30 and 10 ng ml^{-1} of KABI 2161. When samples containing >200 ng ml^{-1} of KABI 2161 were analysed, standard solution concentrations of 320 and 160 ng ml^{-1} were additionally employed.

The eluent for chromatography of the fluorescamine derivatives consisted of acetonitrile–phosphate buffer pH 2.5 ($\mu = 0.1$) (55:45, v/v). For measurements of underivatized prodrug in some of the extraction studies, a mobile phase consisting of methanol–phosphate buffer pH 2.5 ($\mu = 0.1$) (40:60, v/v) was used.

Procedure

Standard curve. The standard curve was prepared by adding 3.0 ml of methylene chloride into four 10 ml centrifuge tubes (glass). Thereafter 1000 μl of the internal standard solution in BTB (32 ng ml^{-1}) was added to each tube followed by the addition of 1000 μl of each standard solution. Finally 500 μl of citrated whole blood was added to the tubes. Extraction was performed immediately for about 15 s by turning the tubes gently by hand. It was important to air the tubes by removing the stopper after the first turns. The tubes were centrifuged immediately at 1650 ***g***. After the centrifugation the aqueous phase was aspirated and discarded. Maximum volume of clear organic phase was thereafter transferred into a 10 ml centrifuge tube.

KABI 2161 as BTB-complex was stable for one week at 20°C when stored in the organic phase. Re-extraction of KABI 2161 was performed by adding 1000 μl of

TBAHS 0.05 M to each tube. These were extracted for 30 min in a mechanical shaker and centrifuged. The upper aqueous phase was transferred to a 10 ml centrifuge tube or directly into the Wisp vial. The pH was adjusted by adding 1000 μl of phosphate buffer pH 7.5, $\mu = 1$. Derivatization took place by rapid addition of 500 μl of fluorescamine, 0.4%, w/v, followed by immediate Vortex mixing. The fluorescamine derivates of KABI 2161 and the internal standard in this solution had half-lives of about 3 days at 20°C. A 100–200 μl portion of this solution was injected onto the column.

Preparation of whole blood samples. Whole blood samples were prepared by adding 3.0 ml of methylene chloride into 10 ml centrifuge tubes (glass). Then 2000 μl of the internal standard solution in BTB (32 ng ml^{-1}) was added to each tube (64 ng i.s/sample) followed by the addition of 100 μl of 3.8 or 10% sodium citrate solution for dog and rat whole blood samples respectively. The tubes were weighed with glass stopper and the weights were noted. About 0.5 ml whole blood was collected directly into the tubes as described below. Duplicates were always collected. The blood samples were extracted as described under "Standard curve". Thereafter the tubes were weighed with stopper and the amount of whole blood was calculated. The samples were centrifuged and treated according to the same procedure as the standards.

A standard curve was constructed by plotting the peak height ratio of KABI 2161 to internal standard against the concentration of KABI 2161 in the standards. The amount of KABI 2161 per ml whole blood was calculated by dividing the value in ng ml^{-1} obtained from the standard curve with the weighed amount of blood in grams. A calibration showed that 1 g whole blood corresponded to 1 ml.

Results and Discussion

Sample handling

KABI 2161 was designed to be a prodrug of tranexamic acid. As such it was expected to hydrolyse rapidly in whole blood. This was demonstrated in whole blood from different species, as can be seen from Table 1. The half-lives in whole blood from the species studied ranged from 1 to 4 min which demanded a rapid procedure for collection of the whole blood samples and for the isolation of the intact drug.

The whole blood samples from rats were taken from the orbital plexus by means of a glass capillary. For dogs the samples were collected from the cephalic vein through a Vacutainer needle. In the present method the samples were collected directly into the tube with the extraction medium (i.e. methylene chloride and BTB containing internal standard) and extracted immediately. Other prodrug samples have been analysed after freezing at −70°C [2] and extracted under thawing. This was compared to direct extraction of blood from two rats given 50 mg kg^{-1} i.v. of KABI 2161. The samples

Table 1
Stability of KABI 2161 in whole blood

Species	Initial concentration of KABI 2161, 10^{-6} moles l^{-1}	Temperature (° C)	$t_{1/2}$ (min)
Rat	2.9	20	1
Human	4.4	37	1.5
Dog	2.1	37	4

were frozen immediately at −70°C in dry ice and ethanol, followed by extraction under thawing within 1 h. The frozen samples also contained 100 μl of 20%, w/v, sodium dodecyl sulphate (SDS) [7] which prolonged $t_{1/2}$ from 1 min to 16 min in rat blood at 20°C. Freezing at −70°C gave only 40% recovery when compared to direct extraction.

It was important to keep the sample volume to about 0.5 ml as samples greater than 1 ml gave rise to emulsions. Insufficient phase separation increased the risk of obtaining disturbing peaks in the chromatogram. Citrate was added to the extraction medium as an anticoagulant. About 0.5 ml blood was collected directly into the extraction tubes. The amount of blood was estimated by subsequent weighing. An attempt was made to alter this procedure by an initial collection of whole blood in a Vacutainer tube followed by rapid transfer of 0.5 ml whole blood to the extraction tube by means of an Eppendorf pipette. The direct extraction method gave 2–3 times higher values of KABI 2161. Possibly the prodrug was adsorbed to the pipette or the Vacutainer tube.

The direct extraction method was chosen because in all tests it gave the highest levels of KABI 2161 in whole blood. If the method has to be applied to larger quantities of clinical samples a less laborious sampling technique with the possibility of storage is necessary.

Extraction

Due to the rapid hydrolysis of KABI 2161 in whole blood a rapid extraction at physiological pH was desirable. A preliminary base extraction from aqueous solution (~1 mg ml^{-1} of KABI 2161) at pH 7.45 $\mu = 1.0$ to methylene chloride ($V_{org} = V_{aq}$, t = 30 min) gave an extraction yield of 66%. In order to increase the degree of extraction, ion-pair extraction with BTB was used. The effect of BTB concentration on the peak–height ratio of KABI 2161/i.s. is given in Fig. 2. As can be seen, a BTB concentration of 0.002–0.003 M is sufficient to obtain a high extraction yield. In the method 0.01 M was used. The explanation for the decrease in the peak–height ratio of KABI 2161/i.s. is that the increase in extraction with increasing BTB concentration is higher for the internal standard, which is probably more bound to blood components than KABI 2161.

The rapid hydrolysis of KABI 2161 in whole blood and the fact that KABI 2161 only exhibits UV-absorption at 210 nm where both BTB and endogenous substances disturb the chromatogram made it difficult to determine the absolute recovery from whole blood. The extraction yield was therefore studied indirectly by measuring the remaining KABI 2161 left in the blood phase by a gas chromatographic method [8] after hydrolysis to tranexamic acid. The remaining whole blood phase after extraction was left at 37°C for 6 h in order to hydrolyse unextracted prodrug to tranexamic acid. The absolute recovery was found to be about 90% at a level of 50 μg KABI 2161 per ml whole blood.

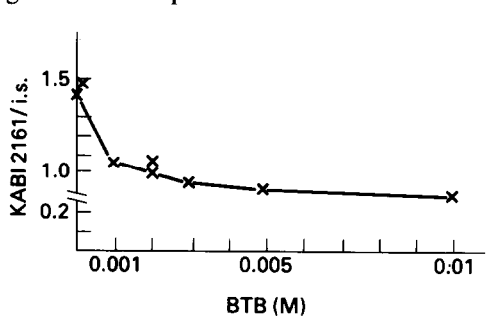

Figure 2
The effect of BTB concentration on the peak–height ratio of KABI 2161/i.s.

No significant difference in the peak–height ratio was observed when the extraction was performed for 15 s or for periods up to 10 min. Due to the rapid hydrolysis in blood a short extraction time was preferred and extraction by hand for 15 s was chosen. This did not give the same risk for emulsion formation as Vortex mixing.

To obtain a quantitative re-extraction of KABI 2161 into an aqueous phase, the use of phosphate buffer of pH about 2 was insufficient (only 43% recovery). However, by adding a competing cation with a high extraction constant with BTB, KABI 2161 should be quantitatively transferred into an acidic aqueous phase. Tetrabutylammonium (as TBAHS) has a very high extraction constant (log K_{ex} = 8.0 with chloroform as organic phase [4]) and gave at a concentration of 0.05 M a quantitative re-extraction of KABI 2161.

Derivatization

It was necessary to enhance the sensitivity by a derivatization step as the prodrug itself only exhibits UV-absorption at 210 nm, where the sensitivity is insufficient ($a_{210} \sim 0.33$) and disturbances from endogenous compounds were severe. Fluorescence detection after pre-column derivatization with fluorescamine was chosen. Fluorescamine selectively reacts with primary amines at a fairly low pH of 7–8 which was advantageous because of the instability of the prodrug. *o*-Phthalaldehyde (OPA) was also examined but was excluded due to solubility problems and the higher pH (about 10) demanded in the derivatization procedure. Further, it has been reported that OPA derivatives of amino acids are relatively unstable with half lives differing from adduct to adduct [9].

Fluorescamine reacts selectively with primary amines, forming intensely fluorescent products. These are shown in Fig. 1 for KABI 2161 and the internal standard. The reaction is rapid and results in one product only. There is, for example, no risk for lactone formation as reported for amino acids [10] as the carboxylic functions of KABI 2161 and the internal standards are esterified.

To determine optimum pH for maximum fluorescence the following study was performed. Solutions of KABI 2161 and of internal standard were prepared in water at concentrations of 80–85 μg ml^{-1}. To 500 μl of these solutions, respectively, were added 1000 μl of phosphate buffers of pH 7–10 and 500 μl of fluorescamine solution. The relative fluorescence was measured after exactly 1 min with a spectrophotofluorometer. The pH given was the apparent pH measured in the reaction mixture. As can be seen from Fig. 3, a pH of >8 was required to obtain maximum fluorescence for both

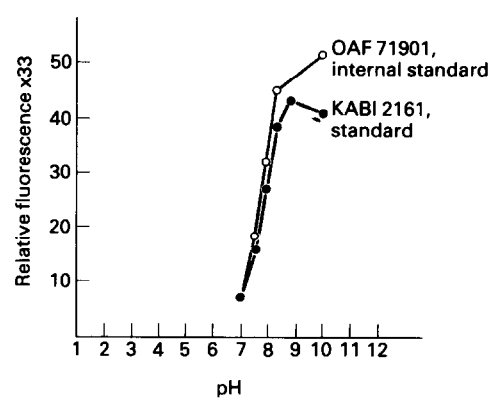

Figure 3
Influence of pH on fluorescence intensity in batch experiments on the formation of fluorescamine derivatives with KABI 2161 and OAF 71901.

substances. To obtain stable derivatives pH 7.5 was chosen in the method. The half-lives of the fluorescamine derivatives at RT were 3 days for both KABI 2161 and the internal standard.

The effect of fluorescamine concentration on fluorescence intensity is given in Figure 4. It can be seen that maximum fluorescence for whole blood samples (100 ng KABI 2161 ml^{-1}) was obtained at 0.3% w/v of fluorescamine. Aqueous solutions of the same concentration required only 0.05% w/v. These fluorescamine concentrations corresponded to molar ratios of reagent: KABI 2161 of between 17 000:1 and 3100:1.

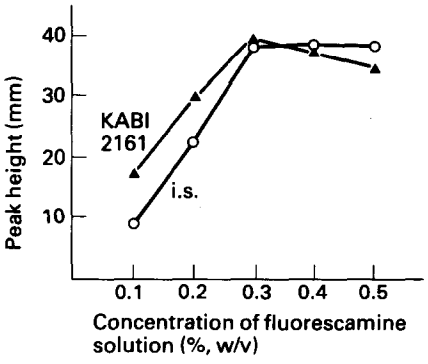

Figure 4
Influence of fluorescamine concentration on fluorescence intensity.

The fluorescence spectra of the fluorescamine derivatives of KABI 2161 and the internal standard were examined using the Aminco Bowman spectrophotofluorimeter, and indicated excitation maxima of 420 nm and emission maxima of 485 nm for both compounds. However, when working with the Schoeffel detector FS 970 which has a deuterium lamp and filters on the emission side, it was found that increased sensitivity was obtained at lower excitation wavelengths and 280 nm was chosen in the method. The sensitivity increased five-fold by changing from λ_{ex} = 390 nm to λ_{ex} = 280 nm.

Quantitative determinations

The minimum concentration that could be determined with the present method was dependent on the volume of blood that was collected and on how much of the organic layer could be collected for further re-extraction into aqueous phase. The limit of detection, defined as a signal to noise ratio of 2, was about 1–3 ng ml^{-1} whole blood when 500 µl blood was used. This was an increase in sensitivity by six orders of magnitude compared to UV detection at 210 nm. The relative standard deviation (n = 10) for the determination of KABI 2161 in whole blood was found to be 4.1% at 300 ng ml^{-1} and 6.9% at 50 ng ml^{-1}. These values include all steps of the analytical method (i.e. extractions, derivatization and chromatography) except for the sampling. The internal standard used was chosen as it was very stable compared to KABI 2161. In human whole blood at 37°C the half-life was about 4 h compared to 1.5 min for KABI 2161. The standard curve of KABI 2161 in the range 10–100 ng ml^{-1} was linear with a slope of 0.0129, an intercept of −0.00027 and a correlation coefficient of 0.9989.

Application to biological samples

The method has been applied to samples from toxicological and pharmacological studies. A chromatogram from an authentic sample from a dog dosed orally with 300 mg kg^{-1} of KABI 2161 is given in Fig. 5.

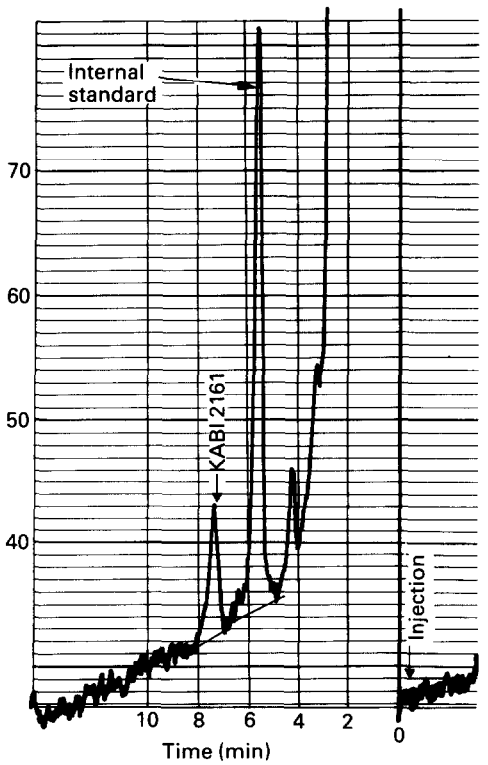

Figure 5
Chromatogram from the analysis of dog blood. Dose: 300 mg kg^{-1} p.o. Time: 2 h after administration. Amount of KABI 2161: 10 ng ml^{-1} whole blood. Eluent: Acetonitrile–phosphate buffer pH 2.5 (55:45, v/v). λ_{ex}: 280 nm. Sensitivity: 0.1 μ A.

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