July 1993 Chem. Pharm. Bull. 41(7) 1261—1265 (1993) 1261

Determination of Auranofin, a Chrysotherapy Agent, in Urine by HPLC with a Postcolumn Reaction and Visible Detection

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Received November 30, 1992

Auranofin [(2,3,4,6-tetra-*O*-acetyl-1-thio-*β*-D-glucopyranosato-*S*)(triethylphosphine)gold(I): AF] is a unique orally active chrysotherapy agent. A HPLC method has been developed for determining AF in urine. The proposed method comprises initial chromatographic separation of AF followed by on-line decomposition by potassium iodide with a released mercapto group undergoing a color-developing reaction with 5,5′-dithiobis(2-nitrobenzoic acid). An aliquot (100 μl) of a urine sample was chromatographed on a YMC AM-302 octadecylsilica column (4.6 mm i.d. × 15 cm, ambient) with a water–methanol (35 : 65) eluent delivered at a flow rate of 1 ml/min. A reagent solution for a postcolumn reaction comprised of 50 μM 5,5′-dithiobis(2-nitrobenzoic acid), 0.3 M potassium iodide and a 50 mM phosphate buffer (pH 7.4), was delivered at a flow rate of 0.5 ml/min. The postcolumn reactor consisted of a poly(tetrafluoroethylene) tube (0.5 mm i.d. × 5 m) at 60 °C. Detection wavelength was 412 nm. The identity of the AF peak was confirmed by a 3-dimensional chromatogram as well as by atomic absorption spectrophotometric analysis of gold in the column effluent. Under the conditions described above, a linear relationship was obtained between peak height and AF concentration in the range 0.1 to 10 μM, with a correlation coefficient of 0.999. The detection limit was 50 nM (*S*/*N*=3 at 0.005 AUFS) and the reproducibility was within 4% for 5 determinations. The AF concentrations in the urine of a rabbit given AF intraperitoneally were determined.

Keywords auranofin; HPLC determination; urine; chrysotherapy agent; postcolumn reaction; visible detection

Gold complexes are a well-established class of agents for the treatment of rheumatoid arthritis (RA).[1)] However, conventionally available gold complexes such as aurothioglucose and disodium aurothiomalate are poorly absorbed from the gastrointestinal tract and therefore have the disadvantage of requiring administration by intramuscular injection. Auranofin [(2,3,4,6-tetra-*O*-acetyl-1-thio-*β*-D-glucopyranosato-*S*)(triethylphosphine)gold(I): AF], depicted in Fig. 1, is a unique orally administered chrysotherapy agent with immunosuppressive and anti-inflammatory properties.[2)] It is a useful agent in the treatment of RA[3)] and possibly severe bronchial asthma,[4)] and in addition shows significant antitumor activity against some tumor types *in vitro* and *in vivo*.[5)]

Despite the important role of AF in the treatment of RA, little is known about its pharmacokinetics and metabolites. The majority of pharmacokinetic studies have been carried out by administering ^{195}Au-labelled AF and then counting radioactivity, or determining gold concentrations by atomic absorption spectrometry.[6)] The behavior of gold-containing species, intact AF and its metabolites, still remains unknown due to the lack of an adequate method to differentiate between them. With cisplatin [*cis*-diamminedichloroplatinum(II)], a well-known and potent antitumor drug, biodegradation in a biological milieu yields products which have been shown to exhibit different biological activities and pharmacokinetic features from those of the intact species.[7)] This also should be the case for AF. In fact, it has been reported that anti-rheumatoid arthritic efficacy can not be predicted from the total gold level in plasma,[8)] although gold is necessary for the efficacy.[9)]

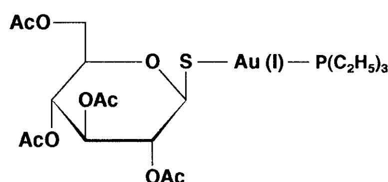

Fig. 1. Structural Formula of AF

In order to clarify the pharmacokinetics of AF in detail, it is necessary to develop an analytical technique to detect and estimate individual species. Of the gold-containing species which may be present in the body, the intact species, that is AF, is by far the most important one, in terms of biological activity. We have made a first attempt at developing a method to specifically determine AF in biological samples. HPLC would seem to be the method of choice since AF is a non-volatile compound. This report describes a method for determining AF in urine by HPLC with a postcolumn reaction and visible detection. The present study was made using urine samples, since it has been suggested that the kidney is a major gold excretory route, when the percentage of AF absorbed from the gastrointestinal tract is taken into account.[10)] Furthermore, blank human urine samples are easily obtainable.

Experimental

Materials AF was obtained, courtesy of the Laboratory of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, and used without any further purification. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) from Nacalai Tesque (Kyoto, Japan) was of a specially prepared reagent grade. Methanol from Katayama Chemical Co. (Osaka, Japan) was distilled once prior to use. Distilled and deionized water was used throughout. Other chemicals were of reagent grade or better and used as received. A stock solution of AF was prepared weekly (1 mM), by dissolving AF in methanol, and was stored under refrigeration. The stock solution was diluted with either a HPLC eluent or 10 mM phosphate buffer (pH 7.4) to give the desired concentrations prior to use. AM-302 (4.6 mm i.d. × 15 cm) and Cosmosil 5CN-R (4.6 mm i.d. × 15 cm) columns were from YMC (Kyoto, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Human urine was obtained from healthy volunteers. Rabbit urine was from male Japanese white rabbits fed with Oriental Yeast RC-4.

Instruments The HPLC system consisted of a Jasco model 880-PU pump (Tokyo, Japan), a Sanuki model DM2U-1026 pump (Tokyo, Japan), a Rheodyne model 7125 sample injector fitted with a 100 μl

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loop (Cotati, CA, U.S.A.), a Jasco model 870-UV variable-wavelength spectrophotometric detector (Tokyo, Japan), a Nippon Denshi Kogyo model U-288 recorder (Tokyo, Japan) and a poly(tetrafluoroethylene) (PTFE) postcolumn reaction coil. The spectrophotometer used was a Shimadzu model 265-FW (Kyoto, Japan). Three-dimensional chromatograms were obtained with a Shimadzu model SPD-M1A spectrophotometric detector. Gold concentrations were determined with a Hitachi model Z-8000 atomic absorption spectrophotometer (Tokyo, Japan).

Analytical Conditions A YMC AM-302 analytical column (room temperature) was eluted with water–methanol (35:65) delivered by the 880-PU pump at a flow rate of 1 ml/min. The reagent solution for postcolumn reaction consisted of 50 μM DTNB, 0.3 M potassium iodide and 50 mM phosphate buffer (pH 7.4), delivered by the DM2U-1026 pump at a flow rate of 0.5 ml/min. The postcolumn reaction coil (0.5 mm i.d. × 5 m) was maintained at 60 °C. Reaction time was estimated to be about 1.5 min based on the retention times of AF with and without the reaction coil. The detection wavelength was 412 nm.

Animal Treatment AF was dissolved at a concentration of 5 mg/ml in 4% polyoxyethylene sorbitan monooleate (Tween® 80), and was administered intraperitoneally to a rabbit (male Japanese white, 2.3 kg weight) at a dose of 20 mg/kg weight. During the first 2 h after AF administration, urine was collected into an ice-cooled tube in 20 min specimens *via* a cannula placed in the bladder through the urethra. The urine samples were immediately centrifuged at 1000 × ***g*** for 1 min at 3 °C to remove insoluble materials, then a portion of the urine was subjected to HPLC to determine AF. Another portion was diluted 10 times or more with 0.1 N hydrochloric acid and then analyzed for total gold concentration by flame atomic absorption spectrophotometry.

Handling of Urine Samples Human urine samples were subjected to HPLC without any pretreatment. Rabbit urine samples were first centrifuged at 1000 × ***g*** for 1 min at 3 °C to remove insoluble substances and were then subjected to HPLC.

Results and Discussion

Strategy of This Study Figure 2 gives an ultraviolet (UV) spectrum of AF. Although AF exhibits a relatively large absorptivity below 220 nm, it would seem impractical to detect AF in biological samples in the vicinity of 210 nm. Therefore, a postcolumn reaction was adopted to detect AF selectively and sensitively. A postcolumn derivation based on a reaction with gold would be preferable, since it might be applicable to the detection not only of intact AF but also gold-containing AF metabolites. Several reagents have been reported for spectrophotometric analysis of gold. These include rhodamine B,[11] di-2-pyridylketoxime,[12] 4-(2-thiazolylazo)resorcinol,[13] furil-α-dioxime,[14] 4-(2-quinolylazo)phenol,[15] phenothiazine[16] *etc.*[17] However, none of them are applicable in our case for the following reasons; some of them react with gold(III) while AF is a gold(I) complex, and the reaction conditions of some agents are not applicable to on-line HPLC postcolumn derivation. Therefore, we decided to use the mercapto group of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosate

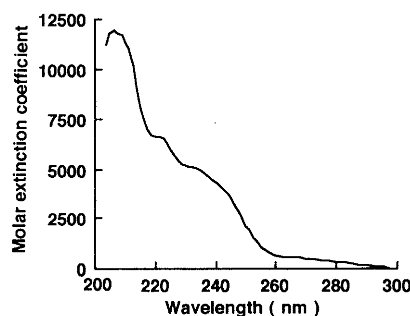

Fig. 2. UV Spectrum of AF

The spectrum was measured using 0.1 mM AF in methanol against a methanol blank.

ligand as a means of detecting AF. Namely, AF might be detected by its on-line decomposition and concurrent derivation of the released mercapto group.

A number of reagents have been developed to modify the mercapto group. Some of them have been used for HPLC analysis of mercapto group containing compounds with postcolumn detection.[18] *o*-Phthalaldehyde (OPA)[19] and 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole (NBD-F)[20] were adopted as fluorogenic reagents. Unfortunately, these reagents have disadvantages in terms of the complexity of HPLC systems and cost. Further, the NBD-F method requires the addition of a 1.5 N hydrochloric acid–50% methanol solution to the flowing solution after the derivation reaction to make products highly fluorescent. Hydrochloric acid is a most unfavorable reagent for HPLC. On the other hand, Ellman's reagent (DTNB)[21] and 6,6′-dithiodinicotinic acid (TNA)[22] have successfully been used for spectrophotometric detection. These reagents give products rapidly through a mercapto-disulfide exchange reaction in aqueous buffers with neutral pH, and therefore, would seem of use. We chose DTNB as a postcolumn derivation reagent since the 5-mercapto-2-nitrobenzoic acid, the colored reaction product of DTNB, has an absorption maximum at a longer wavelength and greater molar extinction coefficient ($\varepsilon = 13600$ at 412 nm)[23] than that of 6-thionicotinic acid, the product of TNA ($\varepsilon = 10000$ at 344 nm).[24]

Chromatographic Behavior of AF on Reversed-Phase Columns First of all, the chromatographic behavior of AF was examined. While AF is insoluble in water, its solubility is higher in organic solvents with a larger polarity. We used two kinds of columns, with nonpolar and polar stationary phases, respectively: the former being a YMC AM-302 octadecylsilica column and the latter a Cosmosil 5CN-R cyanopropylsilica column. Both columns retained AF and gave a single peak. The capacity factor (k') of AF decreased with increasing eluent methanol concentration, indicating that AF was chromatographed in a reversed-phase partition system. The AM-302 column was chosen as an analytical column in subsequent experiments, since it provided a much greater k' of AF than Cosmosil 5CN-R on the same eluent.

Figure 3 illustrates a typical chromatogram of an authentic AF sample on an AM-302 column obtained by direct UV detection at 210 nm. It also illustrates off-line atomic absorption spectrophotometric (AAS) analysis of the gold present in the column effluent. An AAS assay of gold in the effluent revealed that the peak fraction contained gold corresponding reasonably well to the amount of AF loaded onto the column. Further, its three-dimensional chromatogram revealed that the UV spectrum of the peak fraction exactly matched that of the authentic AF given in Fig. 2. These results confirmed the identity of the AF peak.

Release of Mercapto Group from AF Since the AF mercapto group does not directly react with DTNB, we designed a method to decompose AF in the presence of an agent which can coordinate with gold(I) in order to release the mercapto group. Potassium iodide and thiourea were used for this purpose as they appeared to coordinate with AF–gold and did not react with DTNB. Evaluation of the release of the AF mercapto group was carried out by

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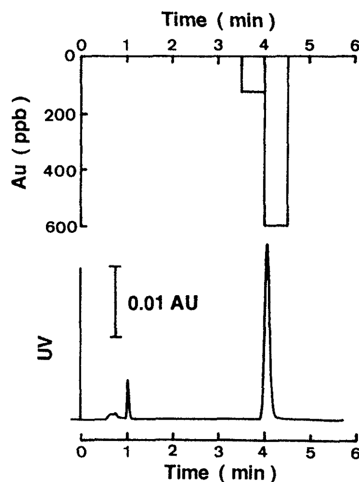

Fig. 3. Chromatograms of AF by On-Line UV and Off-Line Atomic Absorption Spectrophotometric Detection

HPLC conditions are as follows: column, AM-302 (ambient); eluent, water–MeOH (35:65); flow rate, 1 ml/min; detection wavelength, 210 nm. An aliquot (100 μl) of 20 μM AF solution was loaded onto the column and the effluent was collected as 30 s specimens. Gold in the effluent was determined by graphite furnace atomic absorption spectrophotometry.

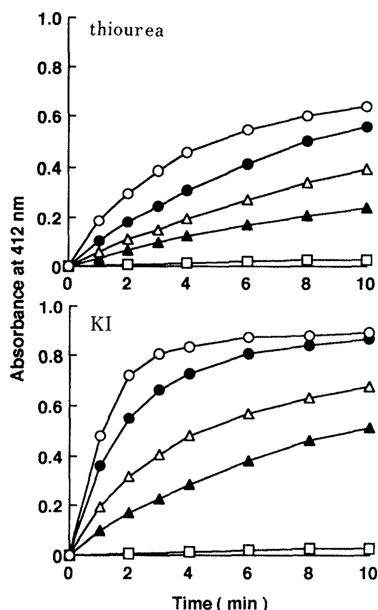

Fig. 4. Release of AF Mercapto Group Mediated by Potassium Iodide or Thiourea

DTNB, potassium iodide, thiourea and working AF solutions were prepared with a 10 mM phosphate buffer (pH 7.4) and a reaction was initiated by mixing 2.8 ml of 0.1 mM AF solution, 0.4 ml of 2 mM DTNB solution and 0.8 ml of a desired concentration of potassium iodide or thiourea solution. The starting concentration of each reagent was as follows: AF, 70 μM; DTNB, 0.2 mM; potassium iodide and thiourea, 0 M (□), 0.03 M (▲), 0.1 M (△), 0.3 M (●), 0.5 M (○). The reaction mixture was allowed to stand at room temperature (about 25 °C) and its absorbance at 412 nm was measured against a blank solution containing 0.2 mM DTNB and potassium iodide or thiourea at the corresponding concentrations.

decomposing AF in the presence of DTNB and by measuring the absorbance at 412 nm, the absorption maximum of 5-mercapto-2-nitrobenzoic acid.[23] As can be seen in Fig. 4, the absorbance at 412 nm increased significantly in the presence of either potassium iodide or thiourea. Further the rate of absorbance increase was greater, the higher their concentrations. These results show that the mercapto group was released by either potassium iodide or thiourea. In subsequent experiments, potassium iodide was used since it gave a greater increase in the absorbance than thiourea at the same concentration.

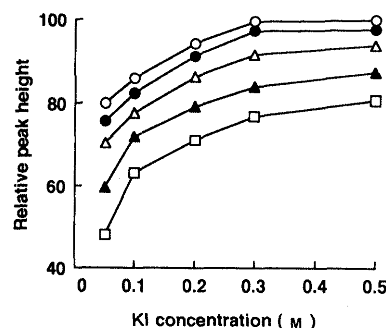

Fig. 5. Effect of Postcolumn Reagent Potassium Iodide Concentration on AF Peak Height at Different Reaction Temperatures

Temperature of the postcolumn reaction: 20 °C (□), 30 °C (▲), 40 °C (△), 50 °C (●), 60 °C (○). Other HPLC conditions are the same as described in the text.

A reaction mixture of AF, DTNB and potassium iodide or thiourea gave a visible spectrum with absorption maxima at 412 nm and this spectrum exactly matched those of the reaction mixtures of DTNB and cysteine, and of DTNB and glutathione. These results ensured that 5-mercapto-2-nitrobenzoate was yielded in this study as the colored compound.

Postcolumn Reaction Conditions The experiment described above showed that AF could be detected indirectly by the absorption of 5-mercapto-2-nitrobenzoic acid. Next, the postcolumn reaction conditions were studied. The postcolumn reaction proposed in this study is composed of two processes: the decomposition of AF, and the reaction between the released mercapto group and DTNB. Figure 4 indicates that both steps proceed rapidly and that it should be possible to run them concurrently in one reaction coil. We decided to use potassium iodide and DTNB in a mixture as our postcolumn reaction reagent. We then examined the effects of potassium iodide and DTNB concentrations and reaction temperature on AF peak height to obtain the optimal conditions for a postcolumn reaction. Chromatographic conditions were the same as described in Experimental.

Figure 5 represents the effect of the varying potassium iodide concentration of the reagent solution on AF peak height at different reaction temperatures. AF peak height increased concurrently with higher potassium iodide concentration and reaction temperature. When the temperature was 60 °C, the AF peak height at 0.3 M potassium iodide was almost identical to that at 0.5 M. The potassium iodide concentration and the reaction temperature were chosen to be 0.3 M and 60 °C, respectively. As for DTNB concentration, the peak height was plotted against AF concentration at different DTNB concentrations (Fig. 6). The AF concentration range, in that the plots were linear, was wider the higher the DTNB concentration, as can be seen in Fig. 6. On the other hand, a significant difference in base line noise was not observed in a DTNB concentration range of 10 to 50 μM, although DTNB itself has absorptivity at the detection wavelength. In the proposed method, 50 μM DTNB was employed.

Determination of AF in Urine Next, we examined the methanol concentration in the eluent and also the detection wavelength. With monitoring at 412 nm, blank urine

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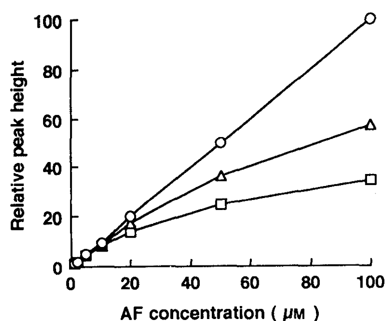

Fig. 6. Relationship between AF Concentration and Peak Height at Different Postcolumn Reagent DTNB Concentrations

DTNB concentration: 10 μM (□), 20 μM (△), 50 μM (○). Other HPLC conditions are the same as described in the text.

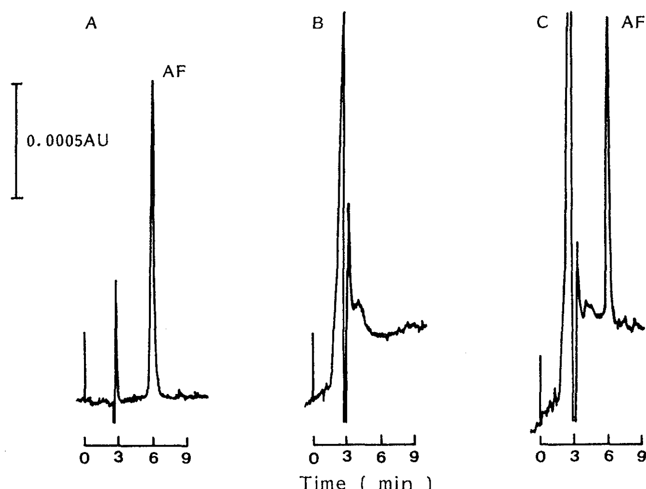

Fig. 7. Chromatograms of Urine Samples

A, 1 μM AF solution; B, a blank human urine sample; C, a urine sample containing 1 μM AF. HPLC conditions as described in the text.

samples did not show any significant peaks except for the column void volume. Therefore, we employed a water–methanol (35:65) eluent and a detection wavelength of 412 nm to determine AF in urine as sensitively and rapidly as possible. Figure 7 represents typical chromatograms of authentic AF and urine samples. The AF peak appeared at 6 min after the injection (A). As mentioned previously, no interfering peaks were found in the blank urine samples (B). The urine samples spiked with AF gave an AF peak, and their heights corresponded to the amounts added (C). Compounds containing the mercapto group are also detectable. Some such compounds in urine must be eluted at the column void volume under the conditions described in Experimental. Cysteine and glutathione gave the peaks at about 3 min. Although the retention time of 2,3,4,6-tetra-*O*-acetyl-1-thio-*β*-D-glucopyranose, a possible metabolite of AF, is not studied, it seems likely that its retention time would be different from that of AF.

A calibration curve for AF in urine made with a peak height showed good linearity in the range 0.1 to 10 μM, with a correlation coefficient of 0.999. The detection limit was 50 nM ($S/N=3$ at 0.005 AUFS). Table I lists the accuracy and the reproducibility of the proposed method. At any given AF level, the concentrations found were in fair agreement with those prepared, and the coefficients of variation were within 4% for five determinations. Under the HPLC conditions obtained in the above experiments, AF in urine samples could be determined accurately and with good reproducibility.

We proceeded to examine the AF stability in urine. Figure 8 gives the time course plots of AF concentration in a human urine sample at different incubation temperatures. While in all cases AF concentration decreased with time, the rate was slower at the lower temperature. When kept at 0 °C, loss of AF was less than 5% after 20 min and no significant degradation of AF was observed for at least 20 min. It is recommended that urine samples be collected

Table I. Accuracy and Reproducibility of the Proposed Method

Added (μM)	Found		
	Mean (μM)	CV (%)	Recovery (%)
1.00	0.98	3.8	98
5.00	4.78	2.2	96
20.0	19.4	3.4	97

AF samples were made up to the given concentration using blank human urine. Analytical data were obtained from 5 determinations. CV = coefficients of variation.

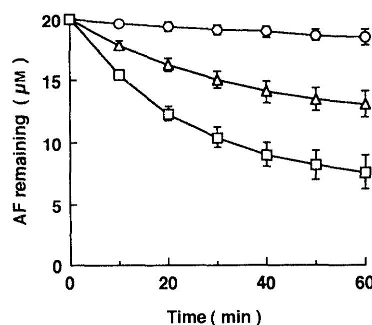

Fig. 8. Stability of AF in Human Urine Samples at Different Temperatures

AF was added to a blank human urine sample to be 20 μM and then incubated at 0 °C (○), room temperature (about 20 °C) (△) or 37 °C (□). An aliquot (100 μl) of the urine was withdrawn at 10 min intervals and immediately subjected to HPLC. Each point is expressed as the mean ± S.D. of triplicate experiments.

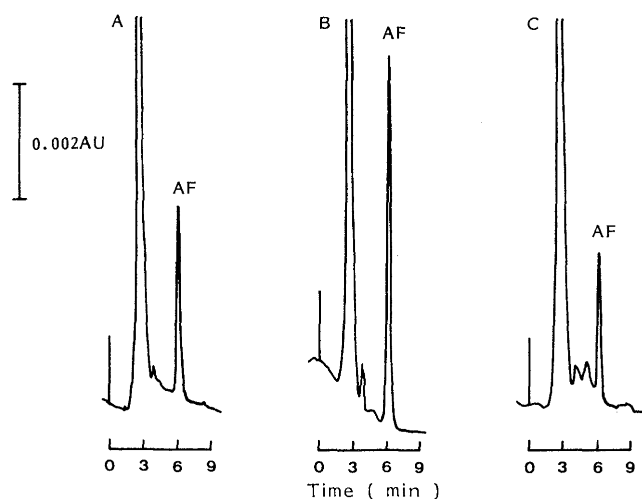

Fig. 9. Chromatograms of Urine Samples Obtained from a Rabbit Given a Single Intraperitoneal Dose of AF (20 mg/kg Weight)

Urine samples were collected as 20 min specimens. Collecting time: A, 20—40 min; B, 40—60 min; C, 60—80 min, after the administration.

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by ureter cannulation, although a cannula was placed in the bladder in the present study and handled rapidly at as low a temperature as possible (about 0 °C) to avoid AF degradation.

AF Concentrations in the Urine Samples of a Treated Rabbit We determined AF concentration in urine samples from a rabbit given AF. Urine samples were collected as 20 min specimens after AF administration, and AF could be detected in three samples, the second (20—40 min), third (40—60 min) and fourth (60—80 min) ones. Chromatograms of these samples are shown in Fig. 9. AF concentrations in those samples were determined to be 2.4, 4.8 and 1.8 μM, respectively. Their ratios to total gold concentrations were 2.8%, 4.5% and 3.8%, respectively, and the amount of AF detected in urine was 0.033% of the dose.

We have developed the first method for determining AF in urine samples by HPLC. The proposed method makes it possible to study the urinary excretion of intact species, a quite new aspect of AF pharmacokinetics, providing a basis for clarifying the pharmacokinetics of AF in detail. Furthermore, this method, with some modifications, seems applicable to other matrices such as stool, bile and synovial fluid, while the present study was made using urine samples. The proposed method should be of use in the pharmacokinetic studies of AF.

Acknowledgement The authors are very grateful to Emeritus Professor Y. Kidani and Associate Professor M. Noji, Nagoya City University, for their kind gift of AF. This work was supported in part by a Grant-in-Aid for New Medicine Development from the Ministry of Health and Welfare of Japan.

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