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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PLASMA ALLANTOIN

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SUMMARY

A new method has been devised for the determination of nanomole levels of allantoin in human plasma. Allantoin was converted into xanthylallantoin, which was chromatographed on a reversed-phase silica gel using a mixture of acetonitrile—water (27:73) as mobile phase. The eluted compound was measured using an ultraviolet detector. The detection limit of the assay for plasma was about 100 ng/ml. This method was applied successfully to the determination of allantoin in human plasma after oral administration of 100 mg of aldioxa.

INTRODUCTION

Aldioxa (aluminium dihydroxyallantoinate), an anti-ulcer drug, is absorbed after hydrolysis to allantoin (I) in the digestive organs [1]. The determination of allantoin at the nanomole level is necessary in human bioavailability studies of aldioxa.

Several reports [2, 3] have been published on determination based on the color reaction of the aldehyde group of glyoxalic acid formed by the hydrolysis of allantoin. Some authors [4, 5] investigated the utilization of the color reaction between 4-dimethylaminobenzaldehyde and allantoin. Recently, Yuki et al. [6] reported colorimetric measurements of the condensation product

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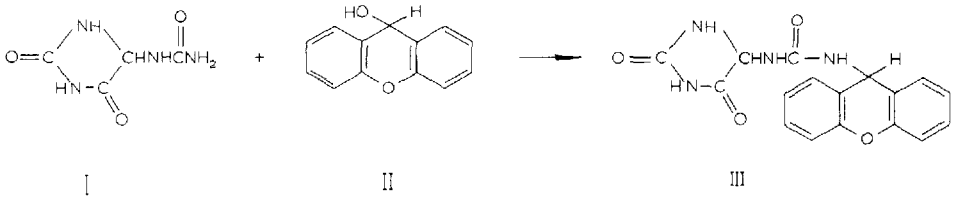

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of allantoin with diacetylmonoximethiosemicarbazide. However, the quantitative analysis of allantoin at nanomole levels in blood has been lacking.

A new method has been devised for the determination of nanomole amounts of allantoin in human plasma. Allantoin was converted with xanthydrol (II) into xanthylallantoin (III), and the derivatized allantoin analyzed on reversed-phase silica gel by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Materials

The standard solution of allantoin (Wako Pure Chemical Industries, Osaka, Japan) was freshly prepared in double-distilled water before analysis. Xanthylallantoin was prepared according to the method of Stewart [7]. The elemental analysis of the compound (as monohydrate) provided satisfactory results. Acetonitrile of UV grade was used. All other solvents and reagents were analytical reagents and used without further purification.

Apparatus

Chromatography was performed on a Shimadzu liquid chromatograph Model 3A, equipped with a variable-wavelength UV detector (Model SPD 2A, Shimadzu, Kyoto, Japan). Injection of samples was automatically performed with an autosampler (Atto, Tokyo, Japan), equipped with a sample bath kept at −1°C by circulation of chilled water. A step gradient of two mobile phases was carried out using a Shimadzu SGR-1A with a time programmer. A 2-μm line filter was inserted between the column and injector port. Plasma samples were homogenized in test tubes (7.5 × 1.0 cm) using a glass bar (0.6 cm diameter) attached to a motor. Evaporation of solvents was carried out with Sarvant Speed concentrator.

Chromatographic conditions

Separation was obtained with a Zorbax ODS column (15 × 0.46 cm; particle size, 5—6 μm; Du Pont, Hitchin, U.K.); the column eluent was monitored at 241 nm at a sensitivity of 0.04 a.u.f.s. A mobile phase of 27% acetonitrile in water was used for analysis of the derivatized allantoin, of which the retention time was 9 min. At 3 min after the appearance of the peak of this compound, the mobile phase was changed to 70% methanol in water for 18 min in order to sweep out interfering substances tightly adsorbed on the column. The mobile phase was again changed to the aqueous acetonitrile, and then the next sample injected after 27 min. The flow-rate was 1.0 ml/min at 43°C.

Extraction and clean-up

A human plasma sample (1.0 ml), which was obtained from fresh blood collected in a heparinized Vacutainer tube, was mixed with methanol (1.5 ml) and allowed to stand overnight in the refrigerator to precipitate most of the plasma proteins. The suspension was centrifuged and the supernant set aside for future use. The precipitate was homogenized with methanol (1.0 ml) to extract the remaining allantoin. After centrifugation, the precipitate containing traces of allantoin was again extracted by homogenizing with methanol (1.0 ml) in the same way. The three methanolic supernatants were combined and evaporated to dryness. The resultant residue was homogenized with methanol (2.5 ml), and the supernatant obtained by centrifugation was evaporated to dryness. Water (1.0 ml) was added to the residue and the resulting solution was washed with three 1.0-ml portions of ether–ethyl acetate (1:1, v/v). The defatted solution was passed through a Sep-Pak C_{18} cartridge (Waters Associates) and the resin washed with water (0.5 ml). The resin was regenerated by washing with methanol (3.0 ml). The combined solution of the eluate and washings was evaporated to dryness, and the resulting residue submitted to derivatization followed by analysis.

Derivatization

To the above residue was added 50% acetic acid in water (0.7 ml), and the solution was cooled to 0°C in an ice-bath. A solution (0.2 ml) of xanthydrol (25 mg) in dimethylformamide (DMF, 1.0 ml) was added. The mixture was allowed to stand at 0°C for 1 h, and was then evaporated to dryness. The resulting residue was suspended in a solution (100 μl) of 50% DMF in water, and diluted with mobile phase (1.0 ml of the aqueous acetonitrile). The diluted suspension was allowed to stand in the refrigerator, and then centrifuged at 8000 *g* for 1 min. An aliquot (0.5 ml) of the suspension was placed in the sample bottle of the autosampler, and an aliquot (50 μl) was injected into the liquid chromatography column.

Quantitative determination

The concentration of allantoin in plasma was directly calculated from a calibration curve. The curve (external standard) of peak height of the derivatized allantoin was obtained by analysis of blank plasma (1.0 ml) to which known quantities (0.5—3.0 μg) of allantoin were added. The curves were prepared for each day of analysis to establish the linearity and reproducibility of the HPLC system.

Drug administration

Five healthy volunteers (23—46 years old, 53—78 kg) were administered a single dose of 200 mg of Bestass (Nisshin Pharmaceutical Co., Tokyo, Japan) which included 100 mg of aldioxa. The drug was administered after overnight fasting, and blood samples were collected in heparinized tubes at 0, 0.5, 1, 2, 3, 5, 8, and 24 h after administration. The volunteers took no food or water until after the collection of the 3-h sample; then their diet was no longer restricted. The samples for the determination of endogenous allantoin were taken in the same way without administration of the drug. The samples were stored at −20°C, and analyzed within three days.

RESULTS AND DISCUSSION

Pre-treatment and derivatization

The plasma sample required a pre-treatment consisting of deproteinization by methanol, elimination of lipid by an organic solvent, and elimination of interfering compounds by a reversed-phase cartridge. Without this pre-treatment an abnormal increase in column pressure was observed, resulting in a shortened column life. The cartridge allowed the passage of allantoin with no adsorption, and could be regenerated by washing with methanol.

The need for repeated extraction by methanol could not be avoided, since a single extraction resulted in a low recovery (about 50%) of allantoin. Although another organic solvent for deproteinization, acetonitrile [8], was used instead of methanol, the recovery was not greatly improved. In addition, extraction from dried plasma (obtained by evaporating plasma under reduced pressure) with methanol was tried. A poor recovery was obtained in this case as well. These facts indicate that plasma has a marked tendency to adsorb allantoin and the allantoin resists desorption from the protein.

Xanthylation of allantoin was carried out by reaction with xanthydrol in 50% acetic acid, using the method previously reported [7]. Since the reagent was used in large excess (about 2000-fold in this case), the reaction mixture contained a large quantity of unreacted reagent with xanthone and xanthene being produced by disproportionation of the reagent [9]. These interfering substances were almost eliminated before injection onto the analytical column. The reaction mixture was evaporated to dryness and the resulting residue suspended in a small volume of DMF (100 μl) that was enough to dissolve the xanthylallantoin. Upon dilution with the aqueous acetonitrile mobile phase, most of the interfering compounds were reprecipitated and were removed by centrifugation.

Use of more than 100 μl of DMF in the above treatment led to the incomplete HPLC separation of xanthylallantoin due to tailing of the DMF. The interfering substances that are reprecipitated upon dilution with mobile phase, should be removed after standing overnight in the refrigerator. Otherwise, gradual precipitation occurs in the sample bottle (cooled at $-1^{\circ}C$) of the autosampler. The precipitate might clog up the needle which sucks the sample solution from the bottle, leading to inaccurate sampling. This multi-step clean-up procedure enabled the analysis of over 300 samples without any abnormal increase in the column pressure and without any deterioration of resolution on the reversed-phase column.

Chromatography

As shown in the chromatogram of a plasma sample spiked with allantoin (Fig. 1c), a symmetrical peak and good separation could be obtained for xanthylallantoin. When plasma samples were analyzed with the single mobile phase of 27% acetonitrile, several peaks continued for more than 60 min after the appearance of the compound. These prolonged peaks interfered with the next injection of sample. This effect could be eliminated by switching to another mobile phase, i.e. 70% methanol. The interfering substances were quickly swept from the column as several peaks appeared after the change of

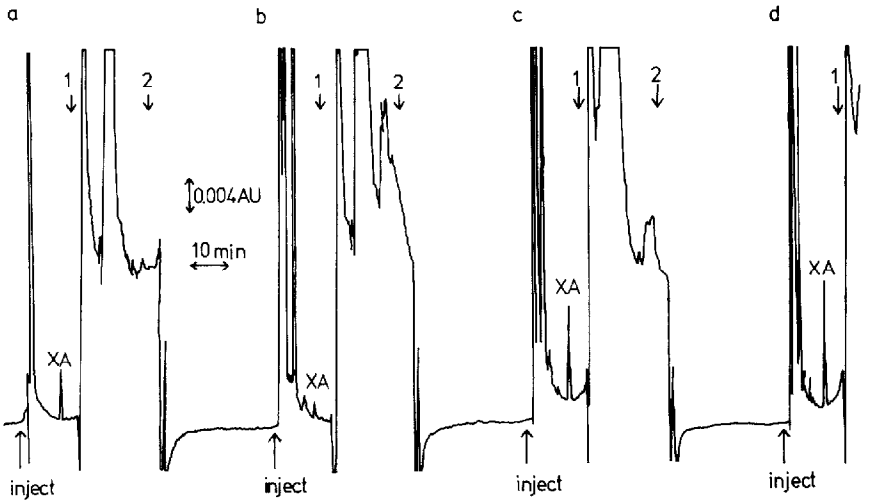


Fig. 1. Continuous analysis of derivatized allantoin. The following samples (1.0 ml) were derivatized to xanthylallantoin (XA) and analyzed under conditions described in Experimental: (a) standard allantoin (1 μmol) in water; (b) blank plasma; (c) plasma spiked with standard allantoin (2 μg); (d) volunteer plasma 1 h after administration of aldioxa. The arrows 1 and 2 indicate the flow start of mobile phases 27% acetonitrile in water and 70% methanol in water, respectively.

the mobile phase (Fig. 1). The switch was performed with a step gradient device connected to the autosampler with a time programmer. One sample could be automatically analyzed in 57 min. A typical successive chromatogram is shown in Fig. 1.

Recovery and precision

Allantoin was added to plasma and then analyzed by the method described above. At the same time, an aqueous allantoin solution was derivatized without the pre-treatment described for plasma. Fifty micrograms of the sample were injected and peak heights of xanthylallantoin measured. The overall recovery of allantoin from plasma was calculated by comparing these peak heights; the results are listed in Table I. The coefficient of variation for identical plasma

TABLE I

RECOVERY ON EXTRACTION AND DERIVATIZATION OF ALLANTOIN

Allantoin added to 1 ml of plasma (μg)	n*	Recovery (% ± S.D.)	Coefficient of variation (%)
0.5	5	98.4 ± 6.0	6.1
1.0	5	104.8 ± 4.6	4.4
1.5	4	93.4 ± 4.9	5.2
2.0	5	96.2 ± 5.4	5.6
3.0	4	103.2 ± 4.7	4.6

*n = number of determinations.

samples varied from 4% to 6%. The precision of the method was evaluated by repeated analysis of plasma with added standard allantoin. The detection limit of the assay was about 100 ng/ml of plasma.

Stability of xanthylallantoin

It was found that the peak height of xanthylallantoin decreased with the passage of the time when the reaction mixture after the derivatization was allowed to stand even at temperatures as low as 0°C. The stability was examined using standard xanthylallantoin (Fig. 2). Acetic acid gradually decomposed the compound even at 0°C, producing an unidentified compound with a retention time of 11.5 min (Fig. 3). However, no decomposition in DMF, which has a high solubility for xanthylallantoin among several solvents tested, occurred at 0°C, although moderate decomposition occurred at ambient temperature. Therefore acetic acid, a solvent for derivatization, was removed by evaporation after derivatization, and the residual xanthylallantoin was dissolved in the mobile phase containing DMF. The resulting solution was cooled at 0°C in an autosampler bottle until it was injected into the analytical column.

Human studies

A small peak in the chromatogram of blank plasma (Fig. 1b) appears at the retention time of xanthylallantoin. The retention time did not differ from that of xanthylallantoin when the analyses were carried out using several mobile phases consisting of acetonitrile and water in several proportions, indicating that the peak is due to endogenous allantoin in the plasma. The endogenous concentration was measured in two volunteers. As seen in Fig. 4, a fluctuation during a day was observed within the range 0.7—1.2 μg/ml of plasma. Archibald [10] and Stahl et al. [11] measured the concentration in individuals using a colorimetric method, and reported the different ranges of 3—6 and 6—11 μg/ml

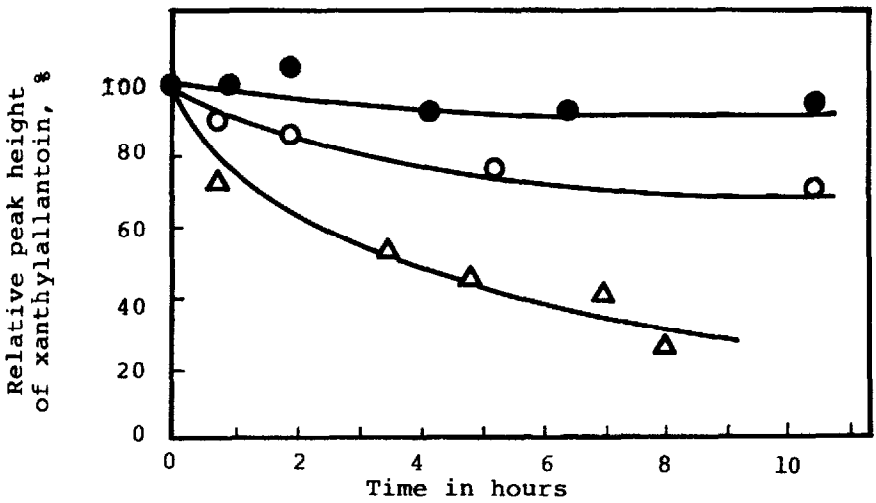
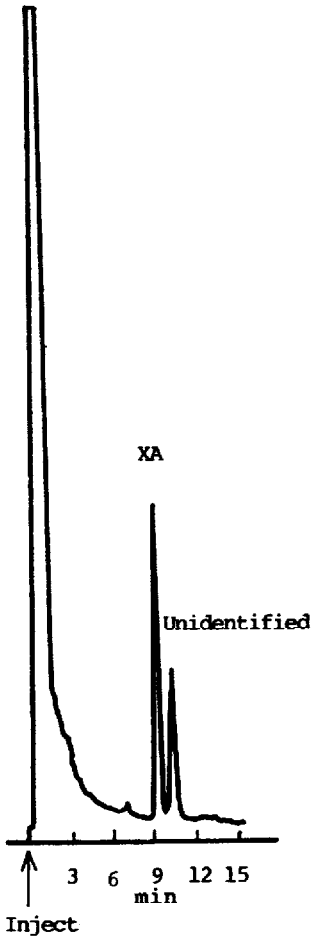


Fig. 2. Stability of xanthylallantoin. Xanthylallantoin (5 μg) in the acetonitrile mobile phase (1.0 ml) containing 7% DMF (•) or 7% acetic acid (○) was allowed to stand at 0°C, and at room temperature in DMF (△).



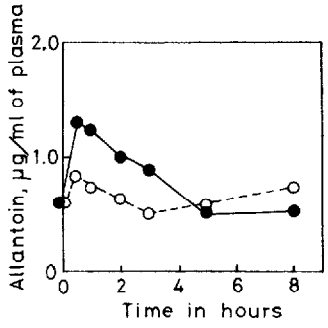


Fig. 3. Production of an unidentified compound from xanthylallantoin. Xanthylallantoin (5 μg) in the acetonitrile mobile phase (1.0 ml) containing 7% DMF was allowed to stand for 24 h at room temperature, and then analyzed.

Fig. 4. Fluctuation of endogenous allantoin in plasma.

of plasma and serum, respectively. These values are much higher than our data. Such a difference may be caused by the different methods used for the determination or by the nature of the specimens from the volunteers.

Allantoin concentration after the administration of aldioxa preparation was measured. The concentration of exogenous allantoin was conveniently calculated by subtracting the allantoin concentration before administration from that after administration, and was then plotted as a function of time (Fig. 5). Allantoin reached its maximal plasma level 1—2 h after administration, and then slowly fell to the pre-administration level 24 h after administration.

This assay with a fully automated system has proved to be reproducible, sensitive, and selective for the determination of allantoin in human plasma at the therapeutic level, and has been used continuously over a year. The sample size required in this procedure makes it suitable for performing pharmacokinetic studies which require multiple biological samples.

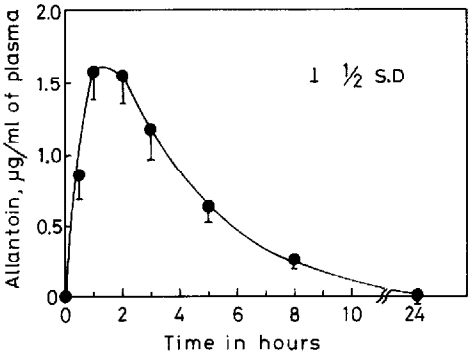


Fig. 5. Plasma concentration of exogenous allantoin after oral administration of 100 mg of aldioxa.

This effective liquid-chromatographic analysis of allantoin modified with xanthydrol indicates that this reagent can be used as a UV-labeling reagent for other drugs having an amide group.

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