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Note

Improved assay procedure for tripamide in blood

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Tripamide, N-(4-aza-*endo*-tricyclo[5.2.1.$0^{2,6}$]-decan-4-yl)-4-chloro-3-sulphamoylbenzamide, is a clinically effective antihypertensive drug [1—3]. Horie et al. [4] have established a method for the determination of tripamide in blood. Single-dose (90 mg) studies in human volunteers showed that tripamide in whole blood was present mainly in red blood cells and that the blood concentration had a relatively long half-life of 9.5 h. However, the method is complicated and time-consuming in pharmacokinetic studies because 50 ml of distilled water are required for haemolysis of each blood sample and 100 ml of diethyl ether for extraction. Thus, there has been no suitable method for monitoring tripamide levels in a routine clinical situation.

This paper describes a simple procedure and a sensitive determination of tripamide in whole blood. Furthermore, the blood concentration of tripamide after oral administration of 10 mg of the drug to human volunteers is also presented.

EXPERIMENTAL

Materials

HPLC-grade acetonitrile, methanol and anhydrous diethyl ether (Wako, Osaka, Japan) were used. The sodium chloride and other chemicals were of reagent grade. Tripamide and the internal standard, 4-(4-chloro-3-sulphamoylbenzoyl)-4-aza-*endo*-tricyclo[5.2.1.$0^{2,6}$]-decane, were prepared in our laboratory as previously reported [5]. [Carbonyl-^{14}C]tripamide was synthesized as described by Nakamura et al. [6]. The radiochemical purity was more than 95% and the specific radioactivity was 31.7 μCi/mg.

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Instrumentation and chromatographic conditions

The high-performance liquid chromatographic (HPLC) system consisted of a Jasco Twincle pump (Jasco, Tokyo, Japan), a Rheodyne 7125 injector with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), and a Jasco Uvidec 100-IV variable-wavelength UV detector.

The HPLC conditions were as follows: column, Zorbax ODS, 250 × 4.6 mm; particle size, 8 μm; mobile phase acetonitrile—water (35:65), 1.3 ml/min; detection, UV at 210 nm.

Procedure

To a 15-ml screw-capped tube (Pyrex, Iwaki, Japan) containing 1 ml of whole blood, 30 μl of internal standard solution (300 ng, acetonitrile) and 1 ml of 3% sodium chloride aqueous solution were added. The samples were sonicated for 20 min using a Ultrasonic cleaner (Branson B-220H, U.S.A.) for haemolysis. After addition of 0.5 ml of 0.1 *M* sodium hydroxide to the samples (approximate pH 10), the extraction was made three times using 6 ml of diethyl ether each time by shaking for 15 min and centrifuging for 5 min at 1000 *g*. The combined ether layers were evaporated at 40°C in a stream of nitrogen gas. The residue was dissolved in 100 μl of acetonitrile by vortexing and sonicating, and after centrifuging for 5 min 20 μl were injected into the HPLC system. The extraction at pH 3 and 7 resulted in low recoveries of 10% and 35%, respectively.

[^{14}C]Tripamide was used for the uptake into red blood cells and the recovery studies. Fresh human blood (1 ml) was incubated with known amounts of [^{14}C]tripamide dissolved in 20 μl of methanol for 30 min at 37°C. For the determination of uptake into red blood cells, the blood sample was centrifuged for 20 min (1200 *g*) and the radioactivity of 50 μl of plasma obtained was measured by liquid scintillation spectrometry using an Aloka Model LSC-635 (Japan Radiation and Medical Electronics). The extraction from whole blood (1 ml) incubated with [^{14}C]tripamide was carried out by the procedure described above. A final extract was dissolved in 100 μl of acetonitrile and the radioactivity of 20 μl of the solution was measured to check the recovery. A part of the acetonitrile solution was also subjected to thin-layer chromatography (Kieselgel GF$_{124}$; Merck, Darmstadt, F.R.G.) with a solvent system of benzene—acetone (1:1), and autoradiography (Fuji X-ray film, Japan) to check the degradation of [^{14}C]tripamide.

Human volunteer studies

Two healthy male volunteers (aged 39 and 40 years, weight 63 and 65 kg) served as subjects. After breakfast, a 10-mg tablet of tripamide, prepared by Merrell Pharmaceuticals (U.K.), was administered orally and 5 ml of blood were taken in heparinized tubes at various intervals. Aliquots of 1 ml of blood were used for extraction and determination of tripamide in duplicate assays. Blood taken before administration was used as a blank sample.

RESULTS AND DISCUSSION

Recovery studies

Recovery studies were performed using whole blood in which [^{14}C]trip-

TABLE I

UPTAKE OF RADIOACTIVITY INTO RED BLOOD CELLS AFTER INCUBATION OF HUMAN WHOLE BLOOD WITH [^{14}C]TRIPAMIDE

[^{14}C]Tripamide added (ng/ml)	Radioactivity in red blood cells (%)	
	Subject A	Subject B
50	>90	>90
200	91.5	91.2
1000	93.4	93.4

amide was incorporated into red blood cells, since after administration tripamide is present mainly in red blood cells [4, 7]. Therefore, the studies on the uptake of [^{14}C]tripamide into red blood cells were carried out in vitro, before determining the recovery from blood.

When [^{14}C]tripamide was incubated at 37°C for 30 min with human blood (1 ml), the uptake of radioactivity into red blood cell was greater than 90%, as shown in Table I. This indicates that the 30-min incubation of blood with tripamide is suitable for determination of recovery. Aliquots (1 ml) of blood were incubated with 50, 200 or 1000 ng of [^{14}C]tripamide, haemolysed and extracted as described. The recovery of radioactivity was 81.4 ± 0.89% (mean ± S.E., $n = 6$).

Additionally, no degradation product of [^{14}C]tripamide was detected by thin-layer chromatography—autoradiography.

Determination of tripamide in blood by HPLC

Under the specified conditions the retention times of internal standard and tripamide were 14 and 21 min, respectively. The chromatograms of 1 ml of

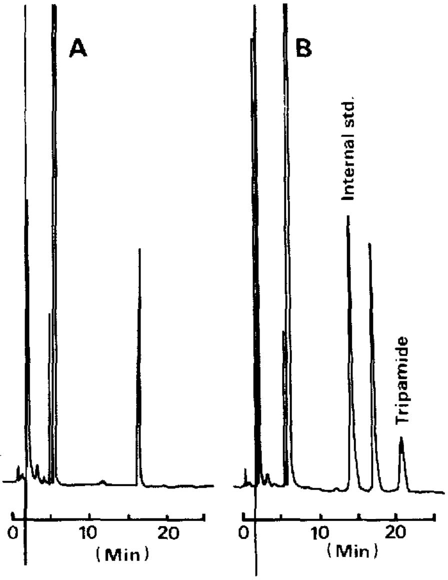

Fig. 1. (A) Chromatogram of extracted blank human blood. (B) Chromatogram of extracted human blood spiked with tripamide (100 ng/ml) and internal standard (300 ng/ml).

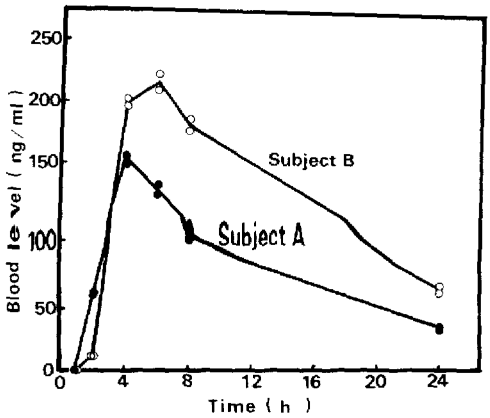

Fig. 2. Tripamide concentration in whole blood after oral administration of a 10-mg tablet of tripamide to healthy human subjects.

whole blood containing 100 ng of tripamide and 300 ng of internal standard are shown in Fig. 1. The blank sample was free from any interfering peaks in the vicinity of the drug and internal standard. The calibration curve from spiked blood (50—500 ng/ml tripamide) exhibited excellent linearity (r = 0.999). The limit of detection using 1 ml of whole blood was 5 ng/ml.

Blood levels of tripamide in human subjects

After oral administration of 10 mg of tripamide to two human volunteers, the blood level of tripamide was measured using the improved assay procedure. As shown in Fig. 2, the analytical method described here had sufficient sensitivity for determination of the blood level; the reproducibility in duplicate assays was also excellent.

After storage of tripamide in blood for three weeks at 4°C, the concentration in all the samples assayed was within the analytical variance of the method.

In conclusion, the present assay method is much more simple and specific than the previous method [4] and will be applied for routine use in pharmacokinetic and clinical pharmacology studies.

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