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# Determination of Aminopyrine and Cyclobarbital from a Skeleton by Radioimmunoassay

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**ABSTRACT:** Aminopyrine and cyclobarbital were detected in the spongy osseous tissue of a skeleton by radioimmunoassay in the concentrations of 12.8 and 3.1 μg/g, respectively.

**KEYWORDS:** toxicology, musculoskeletal system, radioimmunoassay, analgesic, hypnotic, aminopyrine, cyclobarbital

Skeletal remains were found in a forest in Hokkaido, Japan. In the vicinity were two empty drug boxes, one containing aminopyrine, cyclobarbital, phenacetin, and caffeine, and the other glutethimide.

The bones were moderately light and the fatty moiety in the bone had almost disappeared. A compact osseous tissue was crushed easily at some parts of the bone with finger pressure. No tendons or ligaments were found on the surface of the bone. Some nutrient foramina of the bone had been already occupied by roots of plants. Some mummified tissues were attached on the inner surface of the skull as well as in the bone marrow space; however, there was no adipocere formation at any parts of bones. Although there were small bite injuries on some parts of the skeleton, probably made by rodents, no definite wounds were found. From these findings the postmortem interval was estimated to be about two to five years [*1*]. Other skeletal characteristics were morphologically estimated as follows: male, 20 to 30 years old, and 160 to 167 cm tall.

## Materials and Methods

### *Preparation of the Sample*

The spongy osseous tissue in the humerus was removed and ground in a mortar. To 1 g of the bone powder was added 16 mL of 3*N* nitric acid and the mixture was then demineralized at room temperature for 18 h. The pH of the mixture was adjusted to 7 with a saturated sodium hydroxide solution, after which it was homogenized with a Potter-Elvehjem homogenizer and filtered. The filtrate was then concentrated to make about 10 mL of yellowish clear solution. The solution was analyzed by radioimmunoassay (RIA) [*2,3*]. Drug moieties in the

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solution were extracted with chloroform and ethyl ether. The solvent was dried with sodium sulfate. After the organic phase was evaporated in vacuo, some acetone was added and the aliquot was subjected to gas-liquid chromatography (GLC).

*RIA Procedure*

The assay procedures and their application to cadaveric tissue analysis have been described elsewhere [*3–5*]. Briefly, 0.01*M* phosphate-buffered saline (pH 7.6) was used for the dilution of antisera and for the preparation of chemicals and sample solutions. For RIA of pyrazolone derivatives, each tube contained 0.2 mL of cold ligands or the sample solution, 0.1 mL of [$^3$H]antipyrine solution (5000 disintegrations per minute [dpm], 2.13 ng), 0.1 mL of the diluted anti-4-azoantipyrine antibody (1:20) designated as Antibody 1, and 0.1 mL of the buffer. On the other hand, for RIA of barbiturate derivatives, each tube contained 0.2 mL of cold ligands or the sample solution, 0.1 mL of [$^3$H]phenobarbital solution (5000 dpm, 104 pg), 0.1 mL of anti-*p*-succinamidophenobarbital antibody (1:1200) designated as Antibody 2, and 0.1 mL of 1.2% bovine gamma globulin. These mixtures were allowed to stand at room temperature for 2 h, and the separation of free and bound haptens was carried out by a salting-out method with ammonium sulfate. The radioactivity of the bound hapten was measured with a liquid scintillation spectrometer.

*GLC Conditions*

The GLC was performed on a Shimadzu GC-6A instrument equipped with a flame ionization detector. A 3.0-m glass column (0.3 cm in diameter) was packed with 5% SE-30 on Chromosorb W (80-100 mesh) treated with acid washing and dimethyldichlorosilane [*6*]. The temperature of the flame detector and the injection port was set at 230°C. The temperature of the column was maintained at 200°C. The carrier gas (nitrogen) flow rate was adjusted to 40 mL/min.

*Chemicals*

[$^3$H]Antipyrine (specific activity, 0.199 Ci/mmol) and [$^3$H]phenobarbital (5.0 Ci/mmol) were purchased from New England Nuclear in the United States. Aminopyrine and phenobarbital were generous gifts from Fujisawa Pharmaceutical Co., and antipyrine and glutethimide were purchased from Takeda Pharmaceutical Co., Japan. Cyclobarbital calcium was obtained from Shionogi Pharmaceutical Co., Japan, and bovine gamma globulin Fraction II from ICN Pharmaceuticals in the United States. All other reagents were purchased from Wako Pure Chemical Industries, Japan.

## Results and Discussion

Calibration curves, prepared by using known unlabelled antipyrine and aminopyrine or phenobarbital, cyclobarbital, and glutethimide to displace [$^3$H]antipyrine or [$^3$H]phenobarbital from Antibody 1 or Antibody 2, showed that the formation of [$^3$H]antipyrine-Antibody 1 or [$^3$H]phenobarbital-Antibody 2 complex was inhibited competitively and almost linearly, as shown in Fig. 1. The addition of unlabelled antipyrine (15 ng), aminopyrine (30 ng), phenobarbital (0.38 ng), cyclobarbital (8.8 ng), and glutethimide (2900 ng) to each assay system produced a 50% inhibition of binding. The binding affinity of glutethimide for Antibody 2 distinguishes its chemical structure from that of phenobarbital used as a hapten.

Aminopyrine was found present in the bone in the concentration of 12.8 $\mu$g/g and cyclobarbital in the concentration of 3.1 $\mu$g/g.

The gas chromatograms of the pure drug standards and the sample are illustrated in Fig.

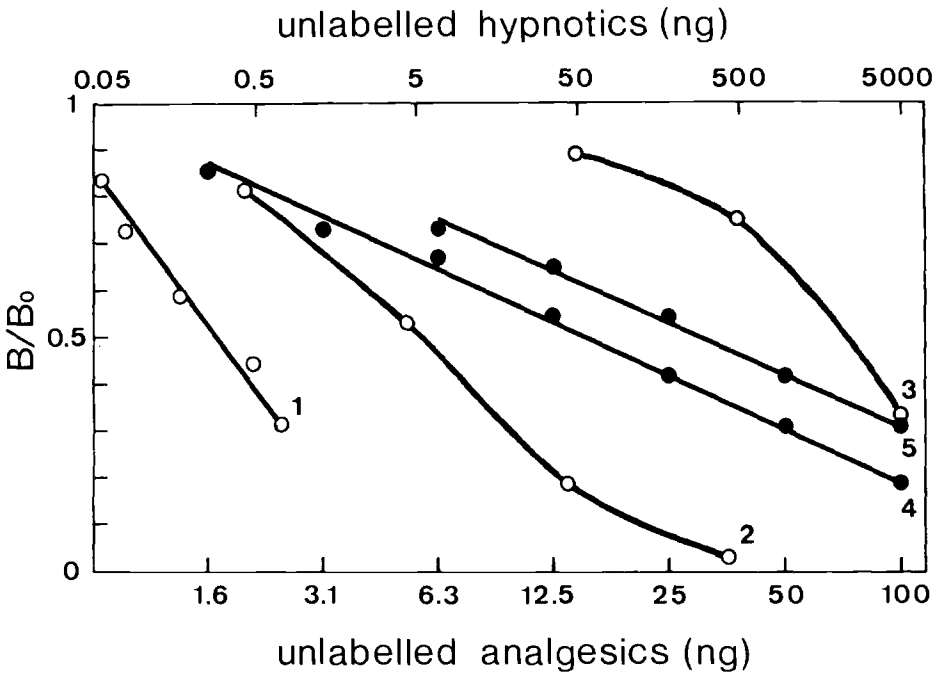


FIG. 1—*Calibration curves for RIA of hypnotics (1: phenobarbital, 2: cyclobarbital, 3: glutethimide) and analgesics (4: antipyrine, 5: aminopyrine).*

2. Only a small peak of aminopyrine from the bone material was found but neither cyclobarbital nor glutethimide was detected, although the latter compound is extracted easily from the sample solution with chloroform.

The Japanese standard body weight corresponding to the skeleton (160 to 167 cm tall) is 55 to 60 kg, and the water content of spongy osseous tissue is approximately 30% [7]. By considering these data jointly with those described above, the total amount of aminopyrine and cyclobarbital for the body weight of the skeleton is estimated to be about 0.49 to 0.54 g and 0.12 to 0.13 g, respectively.

The analysis of drugs from skeletonizing remains has been previously reported [8]. Although interpretations of drug concentrations from bone are not possible at this time, such analyses have potential forensic science applications and should not be ignored by the forensic pathologist and toxicologist.

*Acknowledgments*

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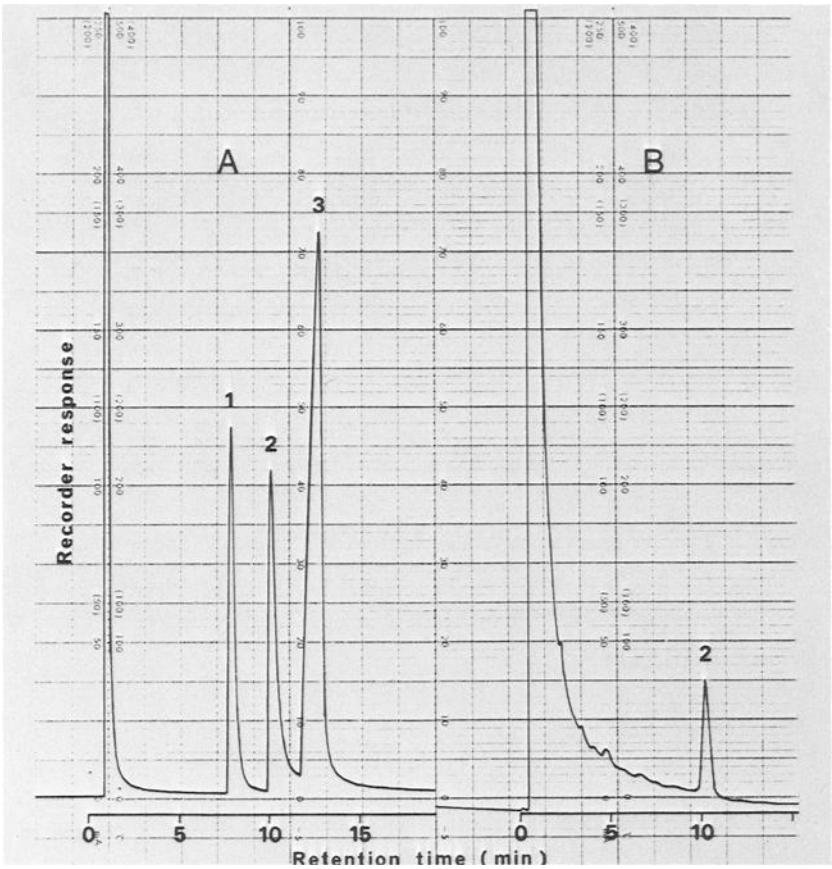


FIG. 2—*Gas chromatograms of the pure standards* (A) *and the sample* (B). *1: glutethimide, 2: aminopyrine, 3: cyclobarbital.*

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