A rapid method for the extraction of angiotensin II from blood

Published methods for the extraction of angiotensin from blood require large amounts of blood and usually elaborate methods of column chromatography for the separation of the hormone from other peptides (Skeggs, Lentz & others, 1967). Recent methods involving radioimmunoassay of angiotensin (Haber, Page & Jacoby, 1965; Page, Haber & Lagg, 1965; Goodfriend, Fasman & others, 1966; Boyd, Landon & Peart, 1967; Catt, Cain & Coghlan, 1967; Catt & Coghlan, 1967; Heffernan, Gillibrand & Prout, 1967; Valotton, Page & Haber, 1967) have greatly increased the sensitivity of measurement and they are also more specific than those using bioassay in the rat which cannot distinguish between angiotensin I and angiotensin II. It is suggested that the following method of extracting angiotensin II from blood would be useful as an adjunct to radioimmunoassay for measuring angiotensin II (and probably angiotensin I) or in conjunction with bioassay procedures using the rat colon (Regoli & Vane, 1964) or the whole rat.

The method to be described is essentially an adaptation of the extraction procedure of Kahn, Skeggs & others (1952) involving the extensive use of centrifuge tubes instead of large pieces of glassware, the substitution of s-butanol for n-butanol and of the final stage of column chromatography using alumina columns with a simple precipitation of angiotensin II from methanolic solution by diethyl ether. The precipitation of angiotensin II by diethyl ether was suggested by Morris & Robinson (1964). The four stages of the present extraction procedure are rapidly performed and the final residue dissolves readily in Krebs-Ringer or Tyrode solution.

Blood (3 ml) is added directly to 6 ml of methanol (AR) contained in round-bottomed centrifuge tubes, 15 ml capacity. The tubes are immediately stoppered with rubber bungs and shaken vigorously. They are then centrifuged at 1000 *g* for 5 min and the supernatant subsequently decanted into centrifuge tubes placed in a metal rack. The volume is then reduced to about 1 ml with a stream of air in a water bath at 40°. This takes 30 to 40 min.

If appreciably larger quantities of blood are taken (up to 50 ml) this initial stage is replaced by one in which the blood is added to four times its volume of laboratory ethanol contained in conical flasks which are stoppered and shaken. The precipitated material is then separated by filtration through Whatman No. 42 paper and the filtrates transferred to round-bottom flasks (250 or 500 ml capacity) and reduced in volume to about 3 ml *in vacuo* at 30° with a rotary evaporator. The material in the flasks is then transferred with Pasteur pipettes to the centrifuge tubes; 2·0 ml of a 1·0M solution of NaCl in 0·2N HCl is then pipetted into the flasks which are scrubbed with a test tube brush for about 1 min. The solution is then added to the first transfer. Another washing and transfer from the flask is then carried out.

When the initial extraction is made in tubes, acidified NaCl solution (5 ml) is added after the initial reduction in volume and 5 ml of diethyl ether added. (No additional acidified NaCl solution is required when the extraction has been carried out in flasks). The tubes are then stoppered and gently shaken for 2 to 3 min. After centrifuging at 100 *g* for 20 s (to resolve emulsions) the upper ethereal layer is removed with Pasteur pipettes and a further extraction then carried out. After the second lot of diethyl ether has been removed air is blown into the tubes for about 20 s to remove any residual ether.

Concentrated hydrochloric acid (0·2 ml) is then added; this is followed by NaCl to give a saturated solution. s-Butanol (chromatographic grade, 2 ml) is pipetted into the tubes which are stoppered and shaken for about 3 min. This is followed by centrifugation at 1000 *g* for 3 min. The upper (s-butanol) layers are then transferred

to centrifuge tubes with Pasteur pipettes. Second similar extractions with s-butanol are then made and the extracts added to the first ones. The tubes are then placed in a water bath at 45° and taken to dryness over about 30 min under a stream of air.

The residues are then partially dissolved in methanol (1·0 ml) and the tubes mechanically agitated for 15 to 20 s on a mechanical agitator (Rotamixer, Model S.N. 536) and then centrifuged at 1000 *g* for 5 min. The clear supernatant is transferred to other tubes with Pasteur pipettes. Diethyl ether (8 ml) is then added, the tubes stoppered and shaken for several seconds and stood in a refrigerator at 4° for 1 h. They are subsequently centrifuged for 2 min at 500 *g* and the methanol–diethyl ether supernatant discarded. The residues are then dried under a stream of air and dissolved in Krebs-Ringer solution (0·3 to 1·0 ml) before testing.

The extracts are free of potassium and contain about a mg of sodium. They are protein-free and contain several mg of peptide as determined by the Lowry reaction (Lowry, Rosebrough & others, 1951) when extracts are made from 50 ml of blood.

The rat colon bioassay method of Regoli & Vane (1964) was used to estimate the replication of the method and the losses of added angiotensin II at the different stages. The overall recovery when 20 ng of angiotensin was added to 50 ml of human venous blood was about 30%. Similar recoveries were observed when this amount of the hormone was added to either dog venous or arterial blood (10 ml). This is comparable with published methods for the extraction of angiotensin II from blood (Osborn, 1966). The replication was excellent, the standard deviation of the population being about $\pm 10\%$ of the mean. When angiotensin II (20 ng) was added before each of the four stages, it was found that most of the losses occurred at the first stage of methanolic (or ethanolic) precipitation. There were negligible losses of angiotensin with the diethyl ether and s-butanol extractions. About 20% was lost in the final stage of diethyl ether precipitation from methanol. There were small cumulative losses in the transfer procedures. Extracts of 50 ml of human venous blood containing no added angiotensin gave contractions of the rat colon only slightly greater than those with Krebs-Ringer solution.

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Metabolism of exogenous cortisol in the rat in various experimental conditions

Cortisol is one of several steroids known to be metabolized by liver microsomal enzymes. The ability to metabolize drugs is lower in very young and old animals (Kato, Vassanelli & others, 1964; Catz & Yaffe, 1967; Kalser, Forbes & Kunig, 1969) and is modified by some drugs like the barbiturates (Conney, 1967). There is also a diurnal rhythm in the activity and metabolism of drugs (Scheving, Vedral & Pauly, 1968; Radzialowski & Bousquet, 1968; Szeberenyi, Szalay & Garattini, 1969).

We now report the half-life of cortisol in the plasma of rats under several experimental conditions. Sprague-Dawley rats of either sex, 150–200 g, were used. The infant rats were 12–14 day old and weighed 45 ± 5 g. The animals were housed at constant temperature (22°) and humidity (60%) in groups of 4–5 animals per cage and kept on a standard diet (Alal 56, Milan). Cortisol hemisuccinate (kindly supplied by Ormonoterapia Richter, Milan) at a dose of 5 or 10 mg of cortisol/kg in 2 ml saline was injected into the tail vein of the animals which were then killed at different times by incision of the carotid arteries. Plasma was collected and tested for cortisol. Experiments were usually made in the morning.

Corticosterone was estimated spectrofluorimetrically (Guillemin, Clayton & others, 1959) and cortisol by the method of Stockham (1963). The daily variations observed in the endogenous corticosterone plasma concentrations of male and female rats are shown in Fig. 1. There is a diurnal rhythm with a fall in the morning and the highest values in the late afternoon.

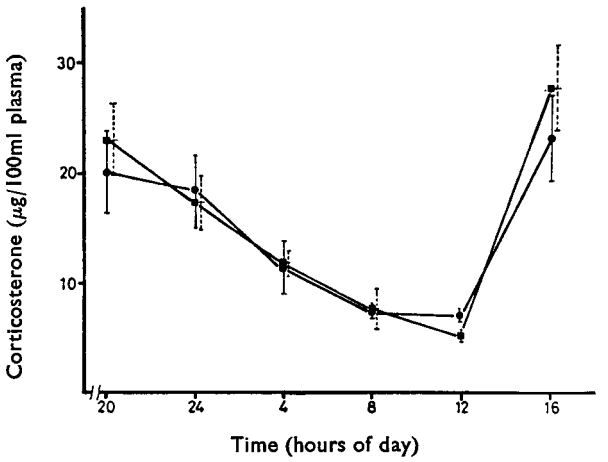

FIG. 1. Mean plasma levels of corticosterone in rats (145 ± 10 g) during the day. ■ --- ■ female, ● — ● male rats.