J Forensic Sci, Jul. 1972, Vol. 17, No. 3

A. P. Phillips,[1] *Ph.D.*, *B. Webb*,[1] *and A. S. Curry*,[1] *Ph.D.*, *F.R.I.C.*, *F.R.C.Path.*

The Detection of Insulin in Postmortem Tissues

The detection of insulin in postmortem tissue from a victim of homicide has been reported [*1*]. The assay involved injection of tissue extracts into mice and reversal of the induced hypoglycemia by glucose. The sensitivity was such that for a successful detection, tens of units of insulin would have to be present at the site of the injection at the time of death. When death has been delayed, activities of insulin about the injection site may be well below this figure [*2,3*]. The development of a radioimmune assay method provides an attractive alternative procedure. In a forensic toxicology laboratory it is inconvenient to keep mice solely for use in an occasional insulin assay and also, the radioimmunoassay has been found to be more sensitive. The principle of the analysis is that of isotope dilution, the extracted insulin being used to compete with iodine-125 labeled insulin for a limited quantity of anti-insulin antibody.

Although the measurement of plasma and serum insulin activities is an established clinical procedure no details of techniques suitable for forensic toxicological purposes were available and hence it was decided to apply the method of Hales and Randle [*4,5*] on postmortem samples of tissue.

Experimental

The method originally used in conjunction with bio assays [*1*], involved extraction in 80 percent ethanol-phosphoric acid, filtration through kieselguhr, and precipitation by ethanolic ether. In our hands, however, this resulted in a fatty solid varying greatly in consistency, usually scarcely soluble in aqueous media, and associated with a very poor insulin recovery when tested by the radioimmune assay. Re-solution and precipitation as the picrate, improved neither solubility nor insulin yield.

In considering the possibility of aqueous extraction as an alternative procedure, dilute acetic acid was chosen as likely to provide a medium stabilizing towards insulin yet strongly inhibitory towards insulinase activity. The latter should be further minimized by refrigeration throughout the extraction.

The molarity of acetic acid was found to be critical, a doubling of the standard 0.25*N* concentration resulting in a considerable decrease in insulin specific activity in the final supernatant.

Attempts at further fractionation by ammonium sulphate precipitation and gel filtration on Sephadex G-50 and G-75 cross-linked dextrans using both column and batch techniques, proved ineffective.

Received for publication 23 June 1971; accepted for publication 21 March 1972.

[1] Home Office Central Research Establishment, Aldermaston, Reading, Berks., England.

Extraction of Insulin from Tissue

Tissue specimens comprising skin, muscle, and fat were excised during postmortem examinations and stored at −20 C. Twenty-five-gram portions, representative of the several layers present, were removed from the frozen tissue with the aid of a surgical saw and while in the process of thawing were subdivided with scissors and placed in the 220-ml flask of a MSE top-drive homogenizer. Where applicable, insulin BP (soluble insulin) was added at this stage, diluted as necessary in 0.25 ml of the Amersham buffer B1. After being allowed to stand for 5 min, 65 ml of cold 0.25*N* acetic acid were added, and maceration performed for 1 min at maximum speed. The homogenate was decanted into 50 ml centrifuge tubes, and after washing out the homogenizer flask with a further 10 ml of acid, the combined extract was allowed to stand in ice water for 5 to 10 min.

The tubes were then centrifuged in the 8 by 50-ml angle rotor of an MSE high speed 25 refrigerated centrifuge, for 10 min at 10,000 rpm (g_{max} 11,900 x g; g_{min} 6,000 x g) at a temperature of 4 C. The supernatant was decanted and centrifuged for 1 h at 15,000 rpm (g_{max} 26,700 x g; g_{min} 13,500 x g). After decanting the supernatant slowly through glass wool (to remove the fatty surface layer), samples were stored at −20 C until assay.

Immediately before assay tissue extracts were thawed slowly. Since the freezing/thawing cycle resulted in the formation of a colloidal precipitate (presumably lipoprotein), this was sedimented by centrifugation for 5 min at full speed in an MSE Minor bench centrifuge. The supernatant was cooled by immersion in ice water. To a 1-ml portion were added 25 μl of 1*M* potassium phosphate buffer pH 7.4, and 85 μl of 2*N* KOH. The pH was monitored with a microelectrode while being adjusted to 7.4 by the addition of KOH or HCl as necessary. Care was taken to maintain the temperature at 4 C during and after the neutralization process. The resulting colloidal precipitate containing approximately 70 percent of the material absorbing at 280 nm, was removed by brief centrifugation and discarded.

Insulin Immunoassay

The method was essentially that recommended by The Radiochemical Centre (Amersham, Bucks) for their Insulin Immunoassay Kit, utilizing the "double antibody" method of Hales and Randle [*4,5*].

Assays were routinely performed in triplicate, and compared with a standard curve derived from insulin BP activities of 0, 10, 20, and 40 μU/ml.

One hundred microliter portions of reconstituted insulin binding reagent were dispensed into glass tubes (inside diameter, 5 by 50 mm), previously siliconized with Repelcote by immersion, draining, and drying at 55 C. The tubes were stored briefly at 4 C until the addition of 100 μl of neutralized tissue extract, either neat or diluted in 0.22*M* potassium acetate buffered with 0.022*M* potassium phosphate pH 7.4 (a simulation of the neutralized extraction medium). Alternatively, to tubes allocated to the standard curve an addition was made of 100 μl of insulin BP appropriately diluted in the 0.22*M* acetate buffer containing 0.5 percent bovine serum albumin. After shaking, the tubes were incubated at 4 C for 4 h. One hundred microliters of ^{125}I-insulin solution (62 μU/ml), in 40 m*M* sodium phosphate buffer pH 7.4 containing 0.6 m*M* sodium thiomersalate and 0.5 percent bovine serum albumin, were added, and incubation continued overnight. The contents of each tube were remixed prior to filtration, when insulin bound to the double antibody complex was retained on a 2.5-cm diameter Oxoid membrane filter held in a glass microanalysis assembly over a vacuum flask. Remnants of the reaction mixture were washed through the filter by two successive 1 ml portions of the 40 m*M* phosphate buffer detailed

above but diluted once with horse serum. After the second wash the filter disk was drained, placed in a glass vial suitable for scintillation counting, and dried at 100 C. Five ml of scintillator cocktail were added (0.4 percent 2,5-diphenyloxazole, 0.01 percent 1,4-di(2-[5-phenyloxazolyl]) benzene in Analar toluene), and the vials were counted for radioactivity in the carbon-14 channel of an Intertechnique SL40 liquid scintillation spectrometer.

Recovery of Insulin Added to Control Tissue Samples

The efficiency of the extraction process was investigated by attempting the recovery of insulin BP added in tenfold dilutions from 10,000—1 mU to 25 g tissue samples. In this experiment the tissue used was from the buttock of a road accident victim, subject A in Table 1. As shown in Table 2, the percentage insulin recoveries were rather variable, but serve to demonstrate a detection limit in the region of the penultimate insulin dilution, that is, 10 mU in 25 g tissue, equivalent to 154 μU per ml extract.

TABLE 1—*Control tissue samples data.*

Subject	Clinical History	Cause of Death
A	No evidence of significant natural disease.	Injuries sustained in a road accident.
B	Established diabetic on 30 U Lente insulin daily. Hospitalized in hyperglycaemic coma. Blood sugar stayed over 1000 mg% in spite of 150 U insulin (i.v.) and 350 U (i.m.) in left thigh 6 h antemortem.	Pulmonary oedema due to diabetes mellitus.

TABLE 2—*Recovery of insulin added to buttock of subject A.*

Added Insulin Equivalent mU/ml extract[a]	Extracted Insulin mU/ml extract	Recovery Percentage (corrected for normal insulin level)
154	7.5	5.0
15.4	1.4	9.2
1.54	0.15	8.8
0.154	0.025	6.5
0.0154	0.012	...
...	0.015	...

[a] 1 ml extract = 0.385 g tissue.

Detection of Insulin in Tissues from Diabetic Subjects

Tissues taken from the cadaver of subject B (Table 1) provided a useful test of the ability of the technique to demonstrate a concentration gradient of insulin from an injection zone. Extraction of a sample taken from the left thigh, the site of a massive injection of soluble insulin 6 h before death, suggested an insulin activity about 100 times greater than that from the control sample of calf tissue, and 35 times that of the right thigh (Table 3).

TABLE 3—*Insulin activities extracted from tissues of subject B.*

Tissue	Dilution of Extract Necessary for Assay	Extracted Insulin mU/ml neat extract
right calf	...	0.018
right thigh	X5	0.058
left thigh	X100	1.96

Discussion

Although recoveries are low, the sensitivity obtained using this technique is considerably above that of bioassays and is suitable for demonstrating concentration gradients of insulin about the site of an injection. As such it is eminently suitable for screening purposes in the context of forensic toxicology.

Summary

A method has been developed for extracting insulin from postmortem specimens of skin and subcutaneous tissue, and has been coupled to a radioimmunoassay. The method was used to demonstrate the site of a massive therapeutic intramuscular injection of insulin.

Acknowledgments

We are most grateful to Peter Pullar, M.D., Royal Hampshire County Hospital, Winchester, for supplying the postmortem specimens without which this study would not have been possible.

References

[*1*] Birkinshaw, V. J., Gurd, M. R., Randall, S. S., Curry, A. S., Price, D. E., and Wright, P. H., *British Medical Journal*, BMJOA, Vol. 2, 1958, pp. 463–468.
[*2*] Binder, Christian, *Acta Pharmacologica et Toxicologica*, APTOA, Vol. 27, Sup. 2, 1969.
[*3*] E. R. Arquilla, personal communication.
[*4*] Hales, C. N. and Randle, P. J., *Lancet*, LANCA, Vol. 1, 1963, p. 200.
[*5*] Hales, C. N. and Randle, P. J., *Biochemical Journal*, BIJOA, Vol. 88, 1963, pp. 137–146.

Central Research Establishment
Aldermaston, Reading
Berkshire, R67 4PN
England