

Chapter 26

Computer Data Handling for the Radiochemical Immunoassay of Insulin

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INTRODUCTION

The concentration of insulin in serum is commonly measured by a substoichiometric isotope dilution technique. The most widely used method is based on the original work of Hales and Randle¹ and is routinely implemented using kits supplied by the Radiochemical Centre.² A known quantity of labelled iodinated insulin is mixed with unknown insulin solution and the two insulins are then allowed to compete for an insufficiency of insulin antibody which forms an insoluble complex. The radioactivity of the isolated precipitate then gives a measure of the extent of dilution of the labelled by the unlabelled insulin and hence the concentration of the latter may be determined. Originally iodine-131 was used to label insulin but more recently the longer-lived and less hazardous iodine-125 has been introduced. This isotope decays by electron capture but also yields low energy internal conversion electrons which may be measured by liquid scintillation counting under conditions similar to those used for tritium. Insulin-antibody complexes are collected on membrane filters which are dried and then suspended in a suitable fluor solution.

CALCULATION OF INSULIN CONCENTRATION

A calibration procedure, preferred by many biochemists, and recommended in the manual supplied with each immunoassay kit,² is to assay a series of standard insulin solutions and plot the experimental data on a curve relating count rate to insulin concentration as shown in Fig. 1 (solid line). Concentrations of unknown insulin solutions are then determined by direct reference to the calibration curve. Most measurements are performed in triplicate and the mean count rate used for subsequent calculations. Thus, for analysis of large numbers of samples, manual computation is both laborious and time consuming.

MATHEMATICAL MODELS FOR COMPUTATION

A first approach to this problem was to attempt a mathematical model fit to the curve shown in Fig. 1. The most straightforward method is a linear fit between adjacent points (Fig. 1, broken line). For good experimental data, deviations from the operator

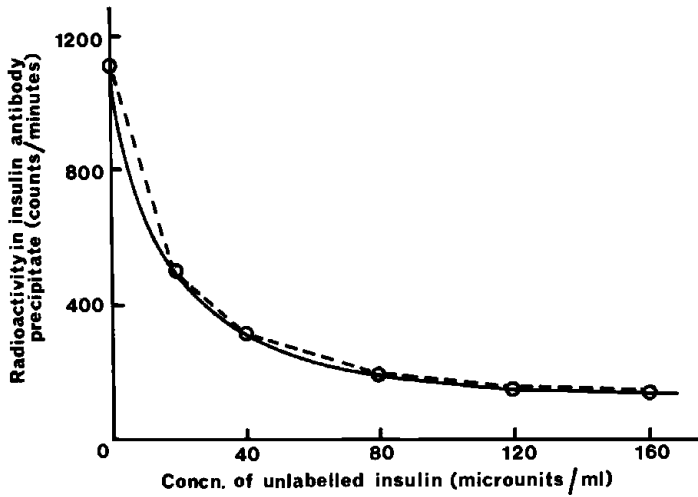


Fig. 1. Calibration curve relating count rate of precipitated insulin-antibody complex to concentration of insulin. (—) operator drawn curve; (-----) linear between pairs fit.

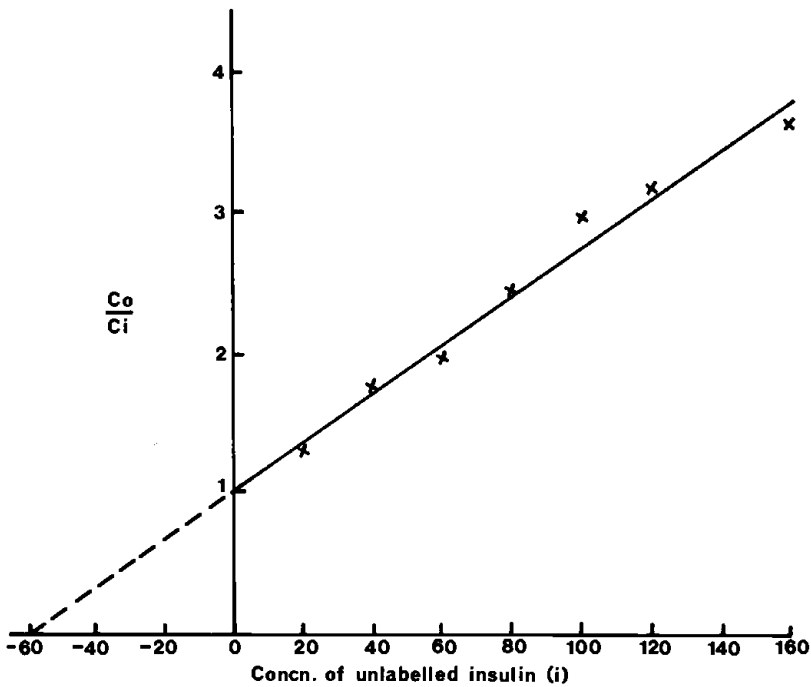


Fig. 2. Calibration curve relating C_0/C_i with insulin concentration.

drawn line are not large. The magnitude of the errors involved in this model could be considerably reduced by using an increased number of standards. The time involved in preparing additional standards was negligible when compared with the time saved by using

a computer to process the results. The model was quite satisfactory if a skilled technician performed the experimental work. However, it was subsequently shown that unacceptable deviations could occur if the experimental calibration results were subject to rather wider variations (see, for example, Fig. 10).

Application of the principles of isotope dilution by Hales and Randle¹ yielded the equation:

$$C_0/C_i = i/i_0 + 1$$

where i and i_0 are the concentrations of unlabelled and labelled insulin respectively and C_0 and C_i are the count rates of the precipitated insulin-antibody complexes when unlabelled insulin concentrations were equal to 0 and i respectively. Theoretically, therefore, there should be a linear relationship between C_0/C_i and i with a slope of $1/i_0$. Unfortunately the isotope dilution principle was not strictly obeyed because the amount of insulin bound by antibody varied with the insulin concentration. Nevertheless, Hales and Randle¹ showed that C_0/C_i plots were linear, at least over the range of insulin concentrations normally encountered, though the slopes of the lines varied with the type of antisera used.

We have confirmed that this linearity holds and have demonstrated that a linear least squares fit to the experimental standard determinations gives a reliable calibration curve which may be used for computational purposes. The line representing the rather poor data displayed in Fig. 10 is shown in Fig. 2. More reliable data, which are most usually available, give a closer fit.

COMPUTER PROGRAMS

For either of the above models, computational processes are similar and may be represented on a simplified flow diagram (Fig. 3). The programs were written in ALGOL 60 and computation was carried out on an Elliott model 903C digital computer having an 8K store. The programs were devised so that either routine or non-routine runs could be accommodated, the technician merely having to supply answers to a few simple questions which are posed by the on-line teleprinter. These are best illustrated by consideration of a specific example where the analyst had prepared eight standards in triplicate and had five samples of unknown insulin concentration of which three had been prepared in triplicate, one in duplicate and one as a single sample. In practice, batches of fifty to a hundred unknowns are dealt with and duplicates or single samples only result from occasional accidents or possibly extremely small samples of serum. Prepared samples are counted for a fixed time and the data collected on punched tape. For convenience, all calculations are performed on total counts rather than c.p.m.

The program is first loaded into the computer and questions are asked which the operator answers on the on-line teleprinter as shown in Fig. 4. (In Figs. 4 to 6, the answers supplied by the operator to questions posed by the teleprinter, have been underlined for clarity). In the routine run illustrated on Fig. 4 all necessary data relating to replication and concentration of the standards, background count, sample number of the first standard etc. are contained on the initial data tape requested in the final demand. For a non-routine run the operator is asked for additional data as shown in Fig. 5. In either case further data are requested for the unknown samples (Fig. 6). Most of these questions are self explanatory. In our laboratory the liquid scintillation counters are multi-user instru-

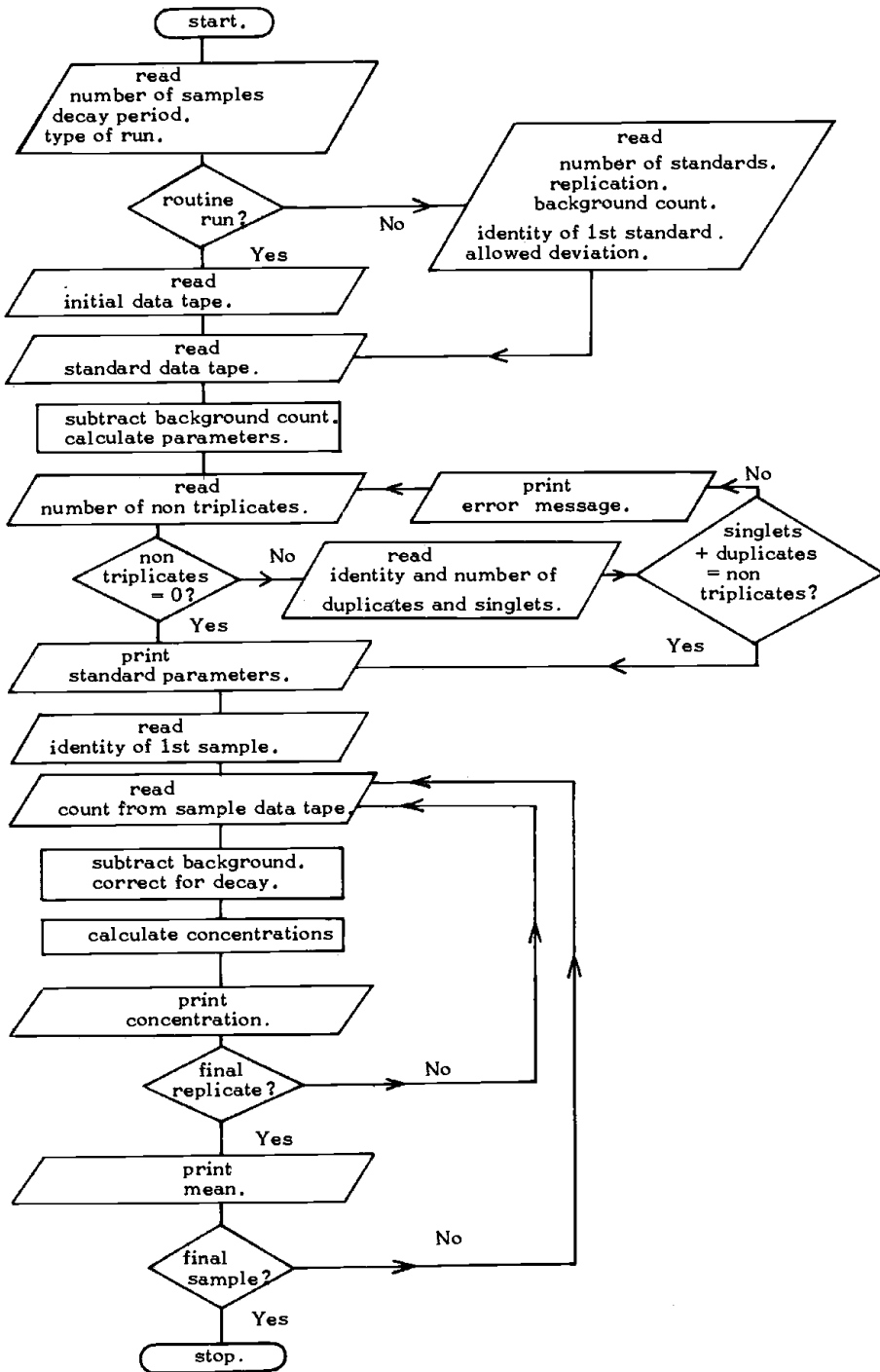


Fig. 3. Flow diagram for computer programs.

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NUMBER OF SAMPLES=5  
NUMBER OF DAYS BETWEEN STANDARD AND SAMPLE COUNT=2  
FOR ROUTINE RUN PRINT 1. FOR NON ROUTINE RUN PRINT 2. 1  
LOAD INITIAL DATA TAPE AND CONTINUE AT 9.  
LOAD STANDARD DATA TAPE AND CONTINUE AT 9.
```

Fig. 4. Format of input for a routine computer run.

```
NUMBER OF SAMPLES=5  
NUMBER OF DAYS BETWEEN STANDARD AND SAMPLE COUNT=2  
NUMBER OF STANDARDS=8  
NUMBER OF REPLICATES=3  
BACKGROUND COUNT=347  
IDENTIFY STANDARD 1,1 1  
STANDARD CONCENTRATIONS=0 20 40 60 80 100 120 160  
ALLOWED PERCENTAGE VARIATION BETWEEN INDIVIDUAL COUNTS AND MEAN=10  
LOAD STANDARD DATA TAPE AND CONTINUE AT 9
```

Fig. 5. Format of input for a non-routine computer run.

```
NUMBER OF SAMPLES NOT IN TRIPPLICATE=2  
NUMBER AND IDENTITY OF SAMPLES IN DUPLICATE 1 1  
NUMBER AND IDENTITY OF SAMPLES AS SINGLETES 1 4  
IDENTITY OF SAMPLE 1,1 25
```

Fig. 6. Format of requests for sample data.

ments and the punched tape output contains much data irrelevant to immunoassay — hence the demand for identification of the first standard. The tape is then automatically searched for this sample before data are taken into the computer store.

Output of results is slightly different for the two programs. For the linear between pairs program (Fig. 7) the triplicate standards are averaged and the mean value used in subsequent computation on unknowns. Where large single deviations within triplicates occur, the program provides for rejection of these results according to a pre-selected maximum deviation from the mean (see, for example, Fig. 5). Such rejections are indicated by the suffix R in Fig. 7, and the mean value recorded is that calculated from the two remaining results.

In the least squares program, standard triplicates are not averaged. All the individual results are fed into the least squares sub-routine for calculation of the best straight line. Net counts and per cent deviations of each point are then printed together with the slope and intercept of the best straight line (Fig. 8). These parameters are then used to calculate

```

          STANDARD COUNTS.
REPL.   1       2       3       MEAN       CONC.
NO
1    15038    15877    15988    15634       0
2    12190    12652    10589 R  12421      20
3    9074     8498     9142     8904       40
4    8031     7724     7977     7910       60
5    6276     6020     6762     6352       80
6    4982     5252     5385     5206      100
7    4731     5151     4673     4851      120
8    4107     5045 R   3596     3851      160

LOAD SAMPLE DATA TAPE AND CONTINUE AT 9.

SAMPLE NO  1    7679    62
              7702    61
                                MEAN= 62

SAMPLE NO  2    4357    135
              4402    134
              4372    135
                                MEAN= 135

SAMPLE NO  3    11123    27
              10544    30
              11109    27
                                MEAN= 28

SAMPLE NO  4    16563    0 OUTSIDE RANGE
                                MEAN= 0

SAMPLE NO  5    3859    155
              3968    151
              3800    154
                                MEAN= 153

FINISH

```

Fig. 7. Output from a computer run using the linear between pairs program.

insulin concentrations for the unknown samples the final results being presented as shown in Fig. 9.

COMPARISON OF MODELS

Experimental data from a run of 25 unknown samples were used to calculate insulin concentration (i) by the traditional operator drawn line and manual calculation, (ii) by computer using the linear between pairs approximation, and (iii) by computer using the least squares fit to a linear C_0/C_i plot. The calibration standards for this comparison were a specially selected *poor* set of data (i.e. the data represented in Fig. 10) chosen so as to emphasize differences between the models. Considering this selection, the results presented in Table 1 show little variation with the method of calculation though in general, method (i) and method (iii) give the closest agreement.

Calibration curves drawn manually through scattered points as shown in Fig. 10 tend to be subjective and no two operators will draw precisely the same curve. A 'best' line through the points of Fig. 10 was obtained via back calculation of C_i values from the C_0/C_i ratios obtained from the least squares best straight line (Fig. 2) and it is clearly seen that this deviates appreciably from the operator's concept of a calibration curve. Thus, in addition to greatly speeding calculation of results, the computer has the added advantage of eliminating operator prejudice from interpretation of calibration data.

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STANDARD NO.	INSULIN CONC.	NET COUNT	C0/CI OBS.	RELATIVE ERROR (PERCENT.)
1 1	0	15038	1.040	2.6
1 2	0	15877	0.985	-2.8
1 3	0	15988	0.978	-3.5
2 1	20	12190	1.283	-6.9
2 2	20	12652	1.236	-11.0
2 3	20	10589	1.476	7.1
3 1	40	9074	1.723	-0.5
3 2	40	8498	1.840	5.9
3 3	40	9142	1.710	-1.2
4 1	60	8031	1.974	-7.4
4 2	60	7724	2.024	-3.3
4 3	60	7977	1.960	-6.7
5 1	80	6276	2.491	1.7
5 2	80	6020	2.597	5.7
5 3	80	6762	2.312	-6.0
6 1	100	4982	3.138	10.5
6 2	100	5252	2.977	5.6
6 3	100	5385	2.903	3.2
7 1	120	4731	3.305	4.1
7 2	120	5151	3.035	-4.4
7 3	120	4673	3.346	5.3
8 1	160	4107	3.807	-2.1
8 2	160	5045	3.099	-25.4
8 3	160	3596	4.348	10.6

GRADIENT= 0.0180 INTERCEPT= 1.0123

Fig. 8. Output of standard data and calibration curve parameters for least squares program.

SAMPLE NO.	NET COUNT	C0/CI OBS.	CALCULATED CONC.	MEAN CONC.	COMMENT
1 1	7679	2.036	57		
1 2	7702	2.030	57		
				57	
2 1	4357	3.588	143		
2 2	4402	3.552	141		
2 3	4372	3.576	143		
				142	
3 1	11123	1.406	22		
3 2	10544	1.483	26		
3 3	11109	1.407	22		
				23	
4 1	16563	0.944	0		OUT OF RANGE
				0	
5 1	3859	4.051	169		OUT OF RANGE
5 2	3968	3.940	163		OUT OF RANGE
5 3	3900	4.009	167		OUT OF RANGE
				166	

Fig. 9. Output of sample data from least squares program.

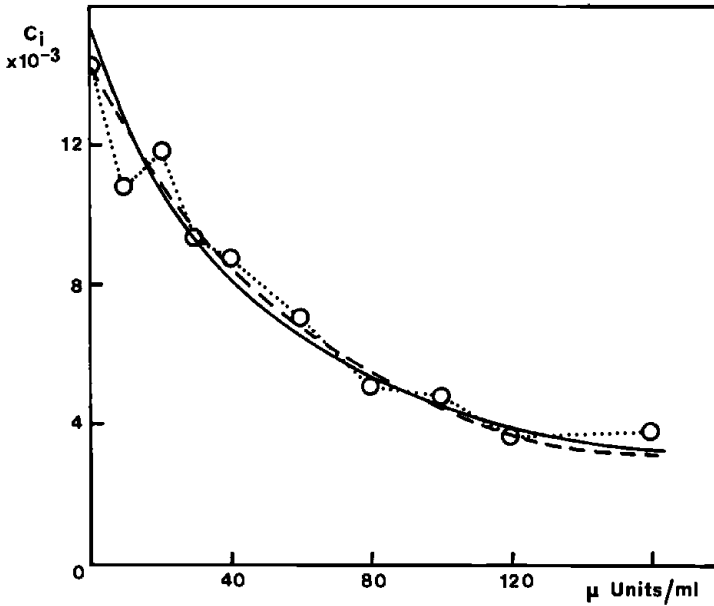


Fig. 10. Comparison of operator drawn line (-----) with least squares fit (——) and linear between pairs fit (.....).

Table 1. Comparison of results calculated via three different methods.

Sample number	Calculated Insulin Concentrations (microunits/ml)		
	Operator drawn (Method (i))	Linear between points (Method (ii))	Least squares (Method (iii))
1	3	3	7
2	7	4	10
3	8	4	10
4	8	5	10
5	8	5	11
6	9	5	11
7	10	5	12
8	11	6	13
9	11	7	13
10	13	7	15
11	19	14	19
12	24	26	23
13	28	29	27
14	39	42	36
15	45	51	43
16	46	52	42
17	58	61	52
18	64	65	57
19	64	65	58
20	81	75	74
21	87	78	80
22	87	78	81
23	99	96	95
24	111	111	111
25	120	120	128

CONCLUSIONS

In this preliminary communication it has been demonstrated that use of a computer can greatly speed calculation of results for insulin immunoassay. Consideration of the calibration function has shown that although it is possible to obtain a good approximation to the conventional calibration curve by a linear between points approximation, a more satisfactory procedure involves a least squares approximation for the best straight line in a plot of C_0/C_i against insulin concentration. Data handling by digital computer also improves reliability by reducing possible errors due to operator prejudice or fallibility.

ACKNOWLEDGEMENT

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REFERENCES

- 1 C. N. Hales and P. J. Randle, *Biochem. J.* **88**, 137 (1963).
- 2 Insulin Immunoassay Kit, The Radiochemical Centre, Amersham, Bucks., England.

DISCUSSION

D. Bowyer: For a given number of points, if one increases the order of the polynomial, one may improve the fit of the curve at the points, but of course between the points one may get a poor fit.

K. L. Evans: Yes, I accept this point but I do not feel it is relevant in the context of our work.

J. W. MacMillan: I agree with Mr. Evans that the discussion of polynomial fitting is irrelevant in the context of this paper since the isotope dilution expression is an exact mathematical description which correlates the data and is, therefore, the preferred one to use for the least squares fit.

F. E. L. ten Haaf: Did you use a least square fit to the absolute or to the relative deviations?

K. L. Evans: We used a least square fit to the absolute deviations.

B. Seaton: I would like to draw attention to the fact that a straight line is merely a first order polynomial and is, therefore, subject to the same general limitations as n th order polynomials. Secondly, it is useful to remember the rule of thumb that when fitting any function having n parameters one needs $2n$ experimental data points to avoid producing the kind of useless curve referred to by Dr. Bowyer. I would also emphasize that an n th order polynomial has $n + 1$ parameters:

$$\text{e.g. } A + Bx + Cx^2 = 2\text{nd order with three parameters}$$

and that it is the number of parameters which must be taken into account.

K. L. Evans: We prefer to use more than four points for the immunoassay calibration.