Journal of Pharmaceutical & Biomedical Analysis
Vol. 8, Nos 8–12, pp. 761–769, 1990
Printed in Great Britain

0731–7085/90 $3.00 + 0.00
© 1990 Pergamon Press plc

A validated HPLC assay for salmon calcitonin analysis. Comparison of HPLC and biological assay*

R.H. BUCK† and F. MAXL

Analytical Research and Development and Quality Assurance, Sandoz Pharma Ltd, CH-4002 Basle, Switzerland

Abstract: A high-performance liquid chromatographic (HPLC) method is described for the assay of salmon calcitonin. The method uses a 5-μm octadecasilyl silica column (100 × 4.6 mm) at 50°C and an initial mobile phase (flow rate 1 ml min^{-1}) comprising 35% of B (1 M tetramethylammonium hydroxide–water–acetonitrile, 8:392:600) and 65% of A (1 M tetramethylammonium hydroxide–water–acetonitrile, 20:880:100) with linear gradient elution over 21 min to a final mobile phase of 57% B; solutions A and B are adjusted to pH 2.5 with phosphoric acid. Detection was by UV spectrophotometry at 210 nm. The method has been shown to be selective, precise and rapid and, in a collaborative study, to give excellent correlation with the results obtained by using the biological assay method of the European Pharmacopoeia. The method, which has been applied successfully to the assay of different batches of salmon calcitonin in bulk drug and in formulated products, is recommended for adoption as the pharmacopoeial assay method.

Keywords: *Salmon calcitonin; high-performance liquid chromatography; biological assay of calcitonin; analysis of calcitonin formulations.*

Introduction

Calcitonin, which is a peptide hormone involved in the regulation of the blood calcium level, was first discovered by Copp *et al.* [1, 2]. Since then, calcitonins of various species have been identified [3–5]. All the calcitonins in principle have the same structure with some differences in the amino acid sequences. The structures of some of the calcitonins are given in Table 1. Several calcitonins, namely salmon, human, porcine, and ($Asu^{1,7}$)-eel, are of clinical importance for use mainly in the treatment of bone diseases.

Some of the calcitonins are described in pharmacopoeias, e.g. salmon and porcine calcitonin in the British Pharmacopoeia [6] and salmon calcitonin in the European Pharmacopoeia (Ph. Eur.) [7]. The pharmacopoeial assay of salmon calcitonin, and its formulations, is an animal response test. This assay measures the lowering of the blood calcium level in rats after intravenous or subcutaneous injection of a sample solution. The hypocalcaemic effect of the sample is compared with that produced by a standard preparation and the potency of the drug is expressed in terms of international units. The animal response assay is very time-consuming, the assay variation is far higher than that of physico-chemical assays and, importantly, it requires animal experiments. Thus there are ethical, scientific and commercial reasons for developing an alternative assay method for this drug.

HPLC is a technique which is widely used in the field of peptide analysis and which has also been applied to the chromatography of calcitonins [8, 9]. In the present work, this technique has been tested for the quantitative determination of salmon calcitonin. An important objective was that a correlation must be established between the potency measured by the biological method and the amount of drug substance measured by HPLC. As calcitonin is obtainable in very high purity the problem of predicting a "potency" by a physicochemical technique is simplified. This work demonstrates that it is possible to replace the animal response assay by an HPLC assay for both salmon calcitonin and its formulations.

---

*Presented at the "Second International Symposium on Pharmaceutical and Biomedical Analysis", April 1990, York, UK.

†Author to whom correspondence should be addressed.

This paper is dedicated to Prof. Dr Franz Lingens on the occasion of his 65th birthday.

Table 1
Peptide sequences of various valcitonins

Salmon calcitonin I

H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2

Human calcitonin

H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2

Porcine calcitonin

H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Ser-Ala-Tyr-Trp-Arg-Asn-Leu-Asn-Asn-Phe-His-Arg-Phe-Ser-Gly-Met-Gly-Phe-Gly-Pro-Glu-Thr-Pro-NH_2

Chicken calcitonin

H-Cys-Ala-Ser-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-Thr-Pro-NH_2

Eel calcitonin

H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-Thr-Pro-NH_2

$(Asu^{1,7})$-eel calcitonin

CH_2-CO-Ser-Asn-Leu-Ser-Thr-NH-CH-CO-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-Thr-Pro-NH_2
| |
CH_2 CH_2
| |
CH_2 CH_2

Experimental

Chemicals and equipment

Acetonitrile and water were of HPLC grade; all other chemicals were of analytical grade. Human, porcine, chicken and eel calcitonin were purchased from Nova Biochem (Läufelfingen, Switzerland). ($Asu^{1,7}$)-eel calcitonin was bought as Elcitonin ampoules from Toyo Jozo Co., Ltd (Shizuoka, Japan). Salmon calcitonin came from the pharmaceutical production of Sandoz Pharma Ltd. The 1st International Standard of salmon calcitonin was obtained from the National Institute for Biological Standards and Control (London, UK).

HPLC analyses were carried out on HPLC systems equipped for automated sample injection, gradient elution, UV detection and automated peak integration.

HPLC method for salmon calcitonin analysis

Mobile phase A was prepared by mixing 20 ml of 1 M tetramethylammonium hydroxide with 880 ml water and 100 ml acetonitrile. Mobile phase B was prepared by mixing 8 ml of 1 M tetramethylammonium hydroxide with 392 ml water and 600 ml acetonitrile. The pH of both mobile phases was adjusted to pH 2.5 with concentrated orthophosphoric acid. The mobile phases were degassed prior to use.

Columns of 100 × 4.6 mm filled with octadecylsilyl silica gel of 5 μm mean particle size were used. Injection volume was 200 μl, flow rate was 1.0 ml min^{-1}, column temperature was set to 50°C. The spectrophotometric detector was set at 210 nm. For routine analysis a linear gradient was run from 35% B to 57% B in 21 min.

Biological assay for salmon calcitonin

The biological assays of salmon calcitonin were carried out according to Ph. Eur. [7]. The assays were performed in three different laboratories. In two laboratories the intravenous method was used, and the other laboratory (laboratory 7) applied the subcutaneous method.

Collaborative study

A collaborative study was performed to compare and validate the two methods with respect to accuracy, precision, and lab-to-lab reproducibility. The study involved three different laboratories on the biological assay and four different laboratories on the HPLC assay. Each laboratory analysed each sample in duplicate or triplicate.

Samples

For the collaborative study, solutions of salmon calcitonin in aqueous buffer of pH 4 were prepared. The concentrations ranged from 30 to 200 i.u. ml^{-1} corresponding to approximately 6 to 40 μg ml^{-1}. The solutions were sterilized by filtration and sealed in ampoules. When the HPLC method was applied to routine quality control analysis, salmon calcitonin drug substance was dissolved in aqueous buffer solution and diluted to a potency of approximately 100 i.u. ml^{-1}. Nasal spray preparations were also diluted to approximately 100 i.u. ml^{-1} with aqueous buffer solution. Injections were assayed without any dilution.

In-house reference standard

Ampoules containing solution for injection from a pharmaceutical production batch were used for this purpose. The in-house reference standard was calibrated against the 1st International Standard of salmon calcitonin by means of biological assays in the three different laboratories. The mean of the assay results was used as the potency of the in-house reference standard. This standard was used for all further calibrations in the HPLC and the biological assay.

The standard, which was stored in the refrigerator, was recalibrated periodically.

Results and Discussion

Reversed-phase HPLC of salmon calcitonin

The retention behaviour of salmon calcitonin on reversed-phase columns was investigated. Retention was strongly dependent on the amount of organic modifier in the mobile phase as shown in Fig. 1. For isocratic elution, the composition of the mobile phase had to be adjusted very precisely to achieve suitable retention. In addition, experience with reversed-phase materials from various suppliers has shown that the amount of organic modifier also depended on the type of column. A more rugged chromatographic separation was achieved by using gradient elution over a narrow range of mobile phase composition. A typical chromatogram of a salmon calcitonin sample is shown in Fig. 2.

Selectivity of the chromatographic system for the separation of various calcitonins was

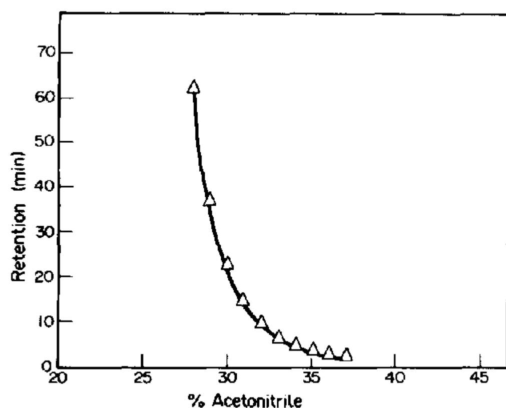

Figure 1
Retention behaviour of salmon calcitonin as a function of the amount of organic modifier.

investigated. Human, porcine, salmon, chicken, eel and ($Asu^{1,7}$)-eel calcitonin were chosen for this purpose. The calcitonins all consist of 32 amino acids and show only minor differences in sequence. For example, chicken and eel calcitonin differ only in two amino acids. Chicken calcitonin contains Ala and Ser in position 2 and 3, while eel calcitonin has Ser and Asn instead. Salmon and eel calcitonin differ in three amino acids. Salmon calcitonin contains Asn, Thr and Ser in positions 26, 27 and 29, where eel calcitonin has Asp, Val and Ala instead. ($Asu^{1,7}$)-eel differs from eel calcitonin by substitution of the cystine in the positions 1,7 by 2-aminosuberic acid. These minor differences in the peptide sequence cause considerable differences in the retention behaviour, so that all the calcitonins can be separated by reversed-phase chromatography. The chromatogram is shown in Fig. 3 and the retention times are listed in Table 2.

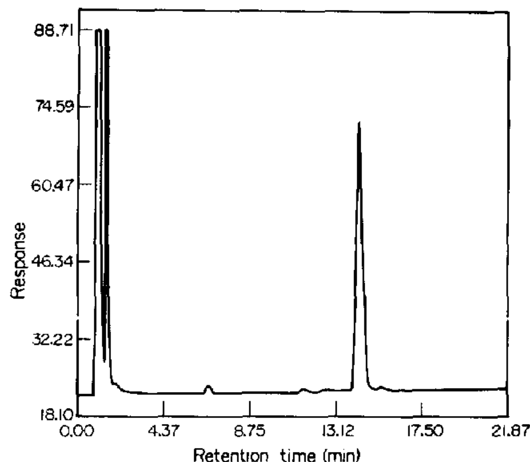

Figure 2
HPLC chromatogram of salmon calcitonin injection. Chromatographic conditions as described under Experimental.

Table 2
Retention time of various calcitonins in reversed-phase HPLC

Type of calcitonin	Retention time (min)
Human	9.21
Salmon	14.55
Eel	16.61
Chicken	17.21
($Asu^{1,7}$)-eel	20.13
Porcine	22.91

Chromatographic conditions as described under Experimental.

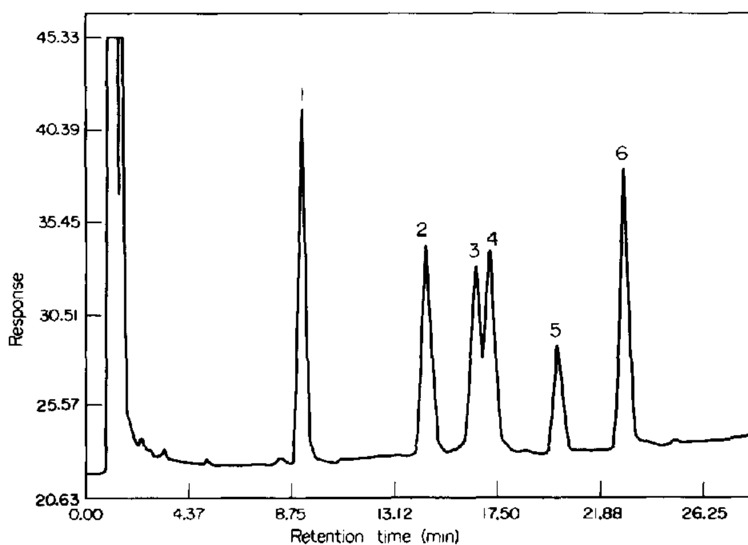

Figure 3
Separation of various calcitonins on reversed phase. 1, human; 2, salmon; 3, eel; 4, chicken; 5, ($Asu^{1,7}$)-eel; 6, porcine. Chromatographic conditions as described under Experimental.

The stability of the sample solution was tested in order to validate the method for use with the autosampling systems. Sample solutions were found to be stable for at least 24 h provided that sterile buffer solutions were used for sample preparation and dilution. Microbial contamination of the buffer solution however leads to a rapid decrease in salmon calcitonin and to formation of degradation products. To avoid microbial contamination sterilized buffer solutions are recommended. Alternatively buffer solutions with a pH of 4 can be used if the buffer solution is prepared several days prior to use. The low pH renders the buffer solution sterile, and microbial degradation of the peptide does not occur.

Correlation between HPLC and biological assay

The correlation between the HPLC assay and the biological assay was established in a collaborative study. A number of samples was analysed by three laboratories using the biological assay and by four laboratories using the HPLC assay. The results of the two methods are given in Tables 3 and 4, and are compared in Fig. 4. A linear regression equation and 95% confidence limits were calculated: the slope of the regression line was found to be 0.92 ± 0.15, which includes the theoretical value of 1.00; the intercept was 6.08 ± 17.61, which includes the theoretical value of 0. The correlation coefficient was 0.9931, which is close to the theoretical value of 1.0000. All parameters of the linear regression equation prove that the assay results of both methods are in good agreement. Furthermore the correlation test shows that both methods are linear in the tested range. Consequently HPLC can successfully be used for the determination of the potency of salmon calcitonin samples.

It should be noted that pure salmon calcitonin samples were used for the correlation studies and that a reliable potency estimation by HPLC requires samples and reference standard to contain only one type of calcitonin and to be essentially free from hypocalcaemically active impurities. In the past, calcitonins of natural or synthetic origin sometimes contained considerable amounts of peptide impurities. Among these impurities there are some which also cause a hypocalcaemic response. It is known that hypocalcaemic activity is not related to one single peptide sequence and that small differences in the sequence do not necessarily affect activity. This can be seen in the sequences for the various calcitonins (Table 1) and for some salmon calcitonin analogues [9, 10]. In the last decade there have been considerable improvements in peptide purification techniques which have resulted in very pure calcitonin products. Based on this prerequisite, a simple and reliable potency estimation can be achieved by HPLC analysis.

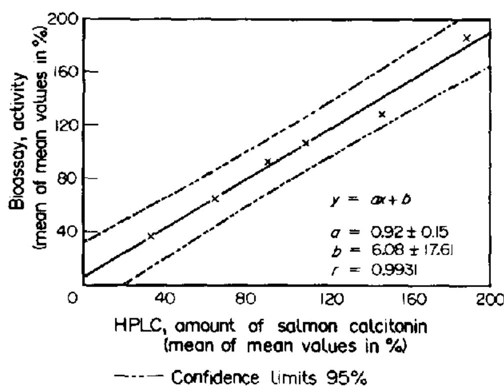

Figure 4
Correlation of biological and HPLC assay results.

Precision of the HPLC and biological assay

The precision of each method was evaluated by calculating the mean intra- and inter-laboratory standard deviations for each sample and

Table 3
Collaborative study: HPLC assay results (in i.u. ml^{-1})

Sample	Laboratory 1		Laboratory 2		Laboratory 3		Laboratory 4		Mean of mean values	Intra-lab SD	Inter-lab SD
	Mean value	SD s_{rel}	Mean value	SD s_{rel}	Mean value	SD s_{rel}	Mean value	SD s_{rel}		S_{1rel}	S_{2rel}
I	187.1	1.6	190.6	0.2	192.8	3.5	186.4	1.3	188.9	0.9	2.5
A	147.7	1.2	146.9	1.1	148.2	0.6	143.3	2.5	146.4	1.1	2.5
L	110.2	0.8	109.1	1.0	111.6	2.7	106.8	0.7	109.2	1.2	2.9
R	92.8	0.4	92.4	1.7	90.8	2.7	86.5	1.9	90.6	2.2	5.5
N	69.7	0.6	65.6	1.3	67.0	0	56.5	1.1	64.5	1.6	15.2
E	35.3	0.8	32.0	0.8	32.4	0.8	32.8	1.3	33.2	3.0	7.5

Table 4
Collaborative study: biological assay results (in i.u. ml^{-1})

Sample	Laboratory 5		Laboratory 6		Laboratory 7		Mean of weighted means	Intra-lab SD	Inter-lab SD
	Weighted mean	Fiducial limits	Weighted mean	Fiducial limits	Weighted mean	Fiducial limits		S_{1rel}	S_{zrel}
I	174.0	—	176.4	163.2–190.6	204.0	180.8–230.3	185.9	15.8	15.2
A	128.0	—	122.6	114.6–131.1	136.3	121.7–152.7	129.1	4.0	9.2
L	102.0	—	102.5	93.5–110.2	118.1	104.9–133.0	107.5	8.7	14.8
R	91.0	—	90.8	85.3– 96.7	95.9	85.5–107.6	92.8	11.6	5.3
N	60.0	—	71.0	67.1– 75.0	64.6	56.1– 70.6	65.2	5.5	14.7
E	40.0	—	38.4	36.0– 41.1	33.2	29.6– 37.2	36.9	7.9	15.7

each assay technique [11]. The results are shown in Tables 3 and 4.

The biological assays gave intra- and inter-laboratory relative standard deviations (RSD) in the range 4–15%. A scatter of this magnitude is common for the biological assay of calcitonin, and for this reason exceptionally wide assay limits of 80–125% and 95% fiducial limits of 64–156% are given in the monographs of the European Pharmacopoeia and the British Pharmacopoeia.

The HPLC assay showed better precision; the intra-laboratory relative standard deviation was in the range 1–3%, and the inter-laboratory RSD ranged from 2.5 to 7.5%, except for sample *N* where an inter-laboratory standard deviation of 15% was recorded. This high value is caused by laboratory 4, which obtained an assay result for sample *N* that was approximately 10% below those obtained by the other laboratories, indicating an outlier. Laboratory 4 shows a tendency to lower assay values for some other samples also, which may have been due to some loss of substance by adsorption on glass-ware, tubings, stainless steel material, etc. during sample preparation or chromatography. The samples contained a very low amount of peptide, e.g. 100 i.u. ml^{-1} salmon calcitonin corresponding to approximately 20 $\mu g\ ml^{-1}$ substance. Such concentrations are in the range of trace analysis, and adsorption of small amounts of peptide onto container surfaces or tubings may lead to a significantly lower assay result. As the intra-laboratory assay precision is very good, such small systematic deviations are easily detected.

In summary, the HPLC assay is more precise than the biological method. However, the outlier value obtained by laboratory 4 demonstrates that the HPLC method might be susceptible to errors and that strict adherence to the procedure is necessary to achieve reliable results.

HPLC in the quality control of salmon calcitonin

Experience was gained with the use of HPLC in the routine analysis of salmon calcitonin drug substance and its formulations. Samples and reference standards of synthetic salmon calcitonin were of very high purity, i.e. biologically active by-products were not present in amounts which could significantly contribute to the biological activity.

The drug substance batches were analysed in two or three different laboratories by biological assay and in one laboratory by HPLC, the in-house reference standard being used for calibration. The results are given in Table 5. For all six batches listed, the HPLC results are within the range of the biological assays. A *t*-test of paired differences [12] showed that there was no significant difference between the HPLC results and the means of the biological assays. This proves that both assay methods are equivalent and that, consequently, HPLC can be used for the determination of potency of salmon calcitonin drug substance.

The results for biological assay in Table 5 display variation that is typical for this method. Variation of this magnitude is normally considered to be unacceptable for the assay of drug substances, because any deviation from the true potency may lead to errors in the content of active drug in the dosage form. To overcome this problem a more precise method is required for the assay of the drug substance. HPLC provides a remarkable improvement in this respect.

A similar comparison of the methods was also made for various batches of salmon calcitonin injection and salmon calcitonin nasal

Table 5
Results of six batches of salmon calcitonin drug substance that were analysed by the biological assay and by HPLC

Batch No.	Biological assay (i.u. mg^{-1})							HPLC assay (i.u. mg^{-1})
	Laboratory 5		Laboratory 6		Laboratory 7		Mean	Laboratory 2
	Results	Fid. lim.	Results	Fid. lim.	Results	Fid. lim.		results
16	4641	3986–5349	5225	4691–5817	n.t.		4933	4903
17	4847	4096–5705	5796	5205–6487	4810	3980–5816	5151	4956
18	4733	4004–5546	5232	4698–5835	4600	3845–5505	4855	4777
19	4807	4284–5377	5011	4393–5726	4682	3913–5612	4833	4833
20	4969	3965–6220	5243	4071–5819	5963	4080–7472	5058	5078
21	4519	3488–5651	5930	5369–6580	4422	3261–5970	4975	4878

n.t. = not tested.

Table 6
Results of salmon calcitonin formulations that were analysed by the biological assay and by HPLC. The results are given in per cent of the declared activity

	Ampuls 100 i.u. ml^{-1} Biological assay		HPLC		Nasal spray 550 i.u. ml^{-1} Biological assay		HPLC
Batch No.	Results (%)	Fiducial limits (%)	results (%)	Batch No.	Results (%)	Fiducial limits (%)	results (%)
76	95.2	65.2–133.8	100.2	84	114.6	90.0–154.9	115.8
77	91.4	81.1–102.6	99.2	85	108.7	95.8–123.6	103.6
78	104.3	88.0–124.7	99.4	86	100.8	73.8–138.4	103.6
79	111.4	97.7–127.3	100.2	87	101.9	87.6–118.5	103.1
80	99.9	82.3–121.1	103.9	88	104.4	83.8–131.9	103.5
81	114.0	102.5–127.2	107.8	89	102.7	88.2–119.4	105.5
82	103.9	76.7–144.3	106.1	90	99.1	82.4–118.8	106.6
83	106.9	94.6–120.7	105.0	91	114.7	98.8–133.7	105.4
84	106.8	81.7–144.3	105.5	92	94.1	83.9–116.9	107.3
85	102.8	87.2–121.3	102.6	93	109.2	92.5–128.9	102.0

spray. The formulations were analysed by HPLC and biological assay in parallel. The results, given in Table 6, are in good agreement. A *t*-test of paired differences shows no significant, systematic difference between the two assay methods. This proves that HPLC can successfully be applied in the quality control testing of salmon calcitonin dosage forms and that it is no longer considered necessary to use a biological assay.

Conclusions

The present study shows that there is a number of aspects which support the use of HPLC in salmon calcitonin analysis. First, the HPLC assay gives an estimated potency which is in good agreement with that obtained with the biological assay. Furthermore HPLC is more precise than the biological method. It has good selectivity, which is demonstrated by the separation of salmon calcitonin from structural analogues. HPLC also is a quick and inexpensive method which is easily automated for unattended continuous analysis and obviates the need to use animal assays. In contrast, the biological test is far more time-consuming and expensive.

Modern quality assurance policy must consider scientific progress and increased ethical awareness in the elaboration of quality control procedures. In the case of salmon calcitonin analysis this means that HPLC should be adopted for the quality control testing of the drug substance and its formulations. At Sandoz Pharma Ltd the following policy will be followed. For drug substance analysis both assay methods, i.e. biological assay and HPLC will be applied. The biological assay will be carried out generally to assure the hypocalcaemic activity of the drug and to estimate roughly the potency. HPLC will be used for the precise determination of the potency which is taken as the stated potency of the batch. For batch compliance, the biological assay result should be within 80–125% of the HPLC value and the fiducial limits of the biological assay must be within 64–156% of the HPLC assay. The ranges 80–125 and 64–156% correspond to the requirements given in the pharmacopoeial monographs for the estimated potency and the fiducial limits, respectively.

Salmon calcitonin formulations will be assayed by HPLC only. HPLC is superior to the biological assay for in-process control and for a precise determination of the amount of drug substance. A biological assay of the product is no longer necessary, because the potency has already been standardized at the drug substance level. This policy permits a decrease in the number of animals used for calcitonin assays at Sandoz Pharma Ltd by 90%. This is a significant contribution to the reduction of animal experiments in the pharmaceutical industry. Moreover, it is proposed that the HPLC assay should be adopted in the pharmacopoeial monographs of calcitonin and its preparations.

References

[1] D.H. Copp, A.G.F. Davidson and B.A. Cheney, *Proc. Can. Fed. Biol. Soc.* **4**, 17 (1961).
[2] D.H. Copp, E.C. Cameron, B.A. Cheney, A.G.F. Davidson and K.G. Henze, *Endocrinology* **70**, 638 (1962).
[3] J.T. Potts Jr, H.D. Niall, H.T. Keutmann, H.B.

Brewer and L.J. Deftos, *Proc. Natn Acad. Sci.* **59**, 1321 (1968).
[4] B.R. Rinker, R. Neher, R. Mayer, F.W. Kahnt, P.G.H. Byfield, T.V. Gudmunsson, L. Galante and I. MacIntyre, *Helv. Chim. Acta* **51**, 1738 (1968).
[5] H.D. Niall, H.T. Keutmann, D.H. Copp and J.T. Potts Jr, *Proc. Natn. Acad. Sci.* **64**, 771 (1969).
[6] The British Pharmacopoeia, p. A164. H.M. Stationery Office, London (1988).
[7] European Pharmacopoeia, Monograph 471. Maisonneuve S.A., Sainte Ruffine, France (1986).
[8] P.W. Lambert and B.A. Roos, *J. Chromatogr.* **198**, 293–299 (1980).
[9] M.L. Heinitz, E. Flanigan, R.C. Orlowski and F.E. Regnier, *J. Chromatogr.* **443**, 229–245 (1988).
[10] D.M. Findlay, V.P. Michelangeli, T.J. Martin, R.C. Orlowski and J.K. Seyler, *Endocrinology* **117**, 801–805 (1985).
[11] G. Gottschalk and R.E. Kaiser, Einführung in die Varianzanalyse und Ringversuche. Bibliographisches Institut, Mannheim (1976).
[12] R. Kaiser and G. Gottschalk, Elementare Tests zur Beurteilung von Messdaten. Bibliographisches Institut, Mannheim (1971).

[*Received for review 5 April 1990*]