ELSEVIER

Journal of Chromatography B, 679 (1996) 129–135

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of penicillin-V in human plasma by high-performance liquid chromatography and solid-phase extraction

W.J.J. Krauwinkel*, N.J. Volkers-Kamermans

Biopharmaceutical Department, Yamanouchi Europe B.V., P.O. Box 108, 2350 AC, Leiderdorp, The Netherlands

Received 16 May 1995; revised 5 December 1995; accepted 5 December 1995

Abstract

A high-performance liquid chromatographic method has been developed for the determination of penicillin-V concentrations between 0.1 and 19 μg/ml in human plasma. Penicillin-V was isolated from plasma by solid-phase extraction on a C_{18}/OH cartridge. The extracts were injected onto a reversed-phase HPLC system. A 125×4 mm C_{18} column was used to separate penicillin-V from its main metabolites, 5*R*- and 5*S*-penicilloic acid and endogenous compounds. The eluent consisted of 66% 0.02 *M* phosphoric acid buffer, to which tetrabutylammonium dihydrogenphosphate and 34% acetonitrile were added. The column effluent was monitored by ultraviolet spectrophotometry at 269 nm. Using this method, penicillin-V concentrations in plasma could be determined with an accuracy between −5.4 and 5.2% and a precision between 0.8 and 1.6%. The method has proved to be reliable and was used in bioavailability studies for the development of a new oral penicillin-V formulation.

Keywords: Penicillins

1. Introduction

Penicillin-V (phenoxymethylpenicillin) and its potassium salt (Fig. 1) belong to the group of small-spectrum β-lactam antibiotics. The chemical structure of the penicillins is characterized by a thiazolidine ring connected to a β-lactam ring, onto which a side chain is attached. Penicillin-V is highly active against Gram-positive cocci such as *Streptococcus pyogenes*, *S. pneumoniae* and non-penicillinase producing *Staphylococcus aureus*, and is a drug of choice in the treatment of infections of the upper respiratory tract. Penicillin-V is mainly excreted by the kidneys, but part of it is converted into the penicilloic acids [1].

Only a few HPLC methods have been published for the analysis of penicillin-V in biological matrices. Lindberg et al. [2] isolated penicillin-V by liquid–liquid extraction with diethyl ether after acidifying the serum. After evaporation of the ether and reconstitution of the residue, the extract was analysed by reversed-phase HPLC. The column effluent was monitored by UV-detection at 215 nm. Their assay was sensitive enough to determine penicillin-V concentrations between 0.125 and 16 μg/ml serum. Extraction recovery was about 75%, but increased at concentrations below 1 μg/ml. Mendez-Alvarez et al. [3] used dichloromethane to extract penicillin-V from serum. Tetrabutylammonium hydrogen sulfate was added as an ion-pairing agent. The organic layer was isolated and evaporated to dryness. The residue was redissolved and onjected into the HPLC system.

*Corresponding author.

0378-4347/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(95)00560-9

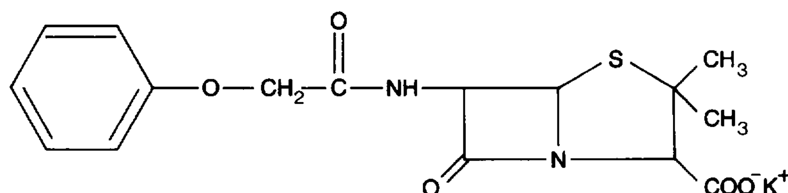

Fig. 1. Chemical structure of penicillin-V, potassium salt.

Detection was performed by UV detection at 208 nm. They were able to determine penicillin-V concentrations between 0.5 and 50 μg/ml serum. Extraction recoveries ranged between 79.4 and 85.4%. For both methods the precision was better than 5%. A method to determine penicillin-V in animal tissues was described by Moats [4]. After protein precipitation with acetonitrile, penicillin-V was isolated using an extensive liquid–liquid extraction. An excellent recovery of 99±3% was reached. The extracts were analysed by HPLC using a polymeric stationary phase and a phosphate buffer (pH 7)–acetonitrile (82:18) mobile phase. Penicillin-V eluted at about 10 min, but because of late eluting components a run time of 30 min was necessary. Detection was performed by UV spectrophotometry at 210 nm. A detection limit of nearly 10 ng/g was obtained, using 15 g of tissue. The assay was not validated for plasma. However, in pharmacokinetic evaluations only a limited amount of plasma is available (usually ≤2 ml). This might lead to an increased detection limit. Moreover, the extensive sample preparation necessary could restrict the number of samples that can be handled on one day. Boison et al. [5] used pre-column derivatisation with 1,2,4-triazole to determine penicillin-G in bovine plasma between 10 and 400 ng/ml. Penicillin-V was added as an internal standard at a level of 300 ng/ml. Before derivatisation, plasma proteins were precipitated using diluted sulfuric acid and sodium tungstate. After dilution with a sodium chloride solution, solid-phase extraction was applied to isolate the penicillins. After elution of the extraction column, a 1,2,4-triazole solution was added to the eluate and the reaction mixture heated at 65°C for 30 min. After filtration, an aliquot was injected onto the HPLC system. The reaction products of penicillin-G and penicillin-V eluted at 4.5 and 5.9 min respectively. UV detection was performed at 325 nm. Using this method they were able to reach a limit of detection of 5 ng penicillin-G/ml. A mean recovery of 91% over a range of 10–400 ng penicillin-G was obtained; the mean accuracy and mean intra-assay precision were 96% and 4.6% respectively (range 20–300 ng penicillin-G/ml). Although the method was not aimed at determining penicillin-V in plasma it is probably suitable for the determination of penicillin-V in plasma using penicillin-G as the internal standard. The method has the advantage of having a low limit of quantitation (LOQ). Unfortunately, an extensive sample clean up is necessary which could restrict the number of samples that can be processed on one day. Although the penicilloic acids are important metabolites of penicillin-V, none of the authors discussed their possible interference.

Three out of four methods used UV detection at low wavelength to enhance sensitivity. Unfortunately, below 220 nm, selectivity generally decreases since many endogenous compounds show UV absorption at these wavelengths and are therefore a possible source of interference. However, the UV spectrum of penicillin-V shows a small maximum at 269 nm due to the phenoxy group. Detection at this wavelength increases the selectivity while validation of the proposed method showed that it is still sensitive enough to quantitate concentrations as low as 100 ng/ml.

Liquid–liquid extraction can be a time-consuming method. Therefore, a solid-phase extraction method was developed for the selective isolation of penicil-

Table 1
Recovery of penicillin-V from human plasma

Concentration (μg/ml)	Recovery (mean±S.D.) (%)
0.203 (n=6)	94.2±1.1
2.03 (n=6)	92.6±1.2
19.5 (n=6)	91.2±0.3

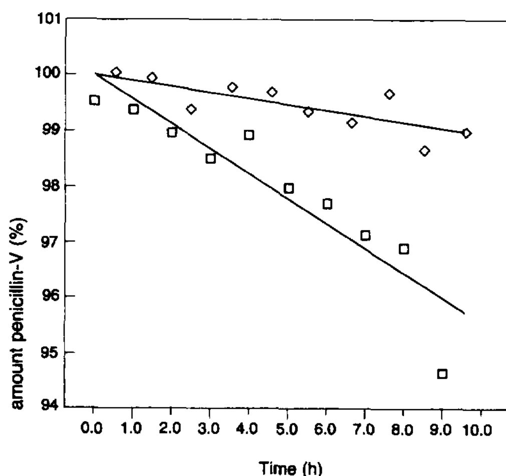

Fig. 2. Stability of penicillin-V at 33°C after sample preparation (squares) and as a solution in 0.0033 M citrate buffer, pH 6.0 (diamonds).

lin-V from plasma. Experience with the assay during the analysis of a large number of samples showed that it was possible to analyse 30–40 samples a day.

2. Experimental

2.1. Column liquid chromatography

The following HPLC system was used: a Spectra Physics Sp 8800 HPLC pump (Thermo Separation Products, Riviera Beach, FL, USA) set at a flow of 0.80 ml/min; a Perkin-Elmer ISS100 auto-sampler (Perkin-Elmer, Norwalk, CT, USA); a Spectra Physics Spectra 100 variable-wavelength UV detector set at 269 nm. The saturation column, pre-column and the analytical column were held at a temperature of 35°C using a column thermostat (Chrompack, Middelburg, Netherlands). A Perkin-Elmer Chrom-3 data system was used for the integration of the chromatograms; peak heights were measured for quantitation. The analytical column used was a 125× 4 mm I.D., stainless steel, prepacked Manucart cartridge containing 5 μm LiChropsher C_{18} as the stationary phase (Merck, Darmstadt, Germany). A 4×4 mm column filled with the same stationary phase (Merck) was used as a pre-column. Between the pump and the auto-sampler, a 100×3 mm I.D. reversed-phase saturation column (Chrompack) was mounted to saturate the eluent with stationary phase. The mobile phase was 0.02 M phosphoric acid buffer–acetonitrile (65:35, v/v) (Labscan, Dublin, Ireland). The phosphoric acid buffer was prepared by diluting 2.3 g (85%, w/w) phosphoric acid (p.a.) (Merck) with 900 ml water; 4 ml of a 1 M solution of tetrabutylammonium dihydrogenphosphate (Aldrich, Milwaukee, WI, USA) was added and the pH was brought to 2.5 with diluted potassium hydroxide (Boom, Meppel, Netherlands). Water was added to make a total volume of 1 l. Water was purified by means of a Milli-Q system (Millipore, Bedford, MA, USA). The run time was 10 min per sample, the penicilloic acids eluted at 3.2 and 3.4 min after injection while penicillin-V eluted after 7.6 min.

2.2. Sample preparation

The solid-phase extraction cartridges (100 mg/ 1 ml C_{18}/OH) used to isolate penicillin-V from plasma were purchased from Analytichem (Harbor City, CA, USA). A 0.017 M citrate buffer, pH 6.0, was prepared by dissolving 3.5 g citric acid monohydrate (p.a.) (Merck) in 900 ml water. The pH was adjusted to 6.0 by adding diluted potassium hydroxide solution, after which water was added to make a total volume of 1000 ml. A 0.003 M citrate buffer was prepared by dissolving 0.7 g of citric acid monohydrate in 900 ml of water. The pH was adjusted to 6.0 by adding diluted potassium hydroxide solution. Water was added to make a total volume of 1000 ml.

The solid-phase cartridges were conditioned by rinsing them with 1 ml acetonitrile and 1 ml of a 0.0017 M citrate buffer pH 6.0. Human plasma samples were diluted by adding 2 ml of the same citrate buffer to 1 ml plasma. The plasma samples were aspirated through the cartridges and the sample vials and cartridges were washed with 1 ml of citrate buffer to which 10% acetonitrile was added. The cartridges were thoroughly dried during 10 min by forcing air through them. Finally the cartridges were rinsed with two volumes of 0.4 ml *tert.*-butylmethyl ether (LiChrosolv, Merck) each and again dried for 1 min. The cartridges were eluted with 1 ml of methanol (Labscan). The methanol was evaporated on a water bath set at 35°C, under a gentle flow of nitrogen for about 5 min. The residues were re-

Table 2
Precision of the assay obtained during validation

Concentration (μg/ml)	Precision, day 1 (%)	Precision, day 2 (%)
0.202 (n=6)	0.8	1.6
2.02 (n=6)	0.8	0.8
18.6 (n=6)	0.5	1.2

dissolved in 100 μl of a 0.003 M citrate buffer, pH 6.0; 50 μl of the sample was injected onto the HPLC system.

2.3. Calibration

A calibration curve consisting of eight different concentrations between 0.1 and 19 μg/ml was constructed. Weighted least-squares regression was used for the calculation of the slope, intercept and the correlation coefficient. $1/C^2$ was used as a weighting factor.

3. Results and discussion

3.1. Recovery

Initially, plasma samples were extracted using Analytichem C_8 SPE cartridges. However, batch-to-batch recovery of penicillin-V varied widely from 34 to 93%. Replacement of the C_8 cartridges by C_{18} cartridges did not improve the recovery, but high and reproducible recoveries were obtained by using C_{18}/OH cartridges.

The strength of the citrate buffer appeared to be important for the recovery of penicillin-V. Initially, the C_{18}/OH cartridges were conditioned by using 1 ml acetonitrile and 1 ml of a 0.003 M citrate buffer, pH 6.0. The plasma samples were also diluted by using the 0.003 M citrate buffer. However, the recovery greatly depended on the type of plasma used. Plasma prepared by adding a citrate buffer to blood gave excellent results, but the use of heparinized plasma resulted in highly variable recoveries (17–60% recovery). Substituting 0.003 M citrate buffer by 0.017 M citrate buffer was sufficient to obtain high and reproducible recoveries, independent of the type of plasma.

Table 3
Accuracy of the assay obtained during validation

Concentration (μg/ml)	Accuracy, day 1 (%)	Accuracy, day 2 (%)
0.202 (n=6)	−3.7	−0.1
2.02 (n=6)	5.2	−3.0
18.6 (n=6)	−5.4	−3.2

The extraction recovery of penicillin-V was determined at 0.2, 2.0 and 19.5 μg/ml (Table 1). Blank human plasma samples were spiked with penicillin-V, extracted and analysed. Injections of the pure standard were used as references. Recovery decreased slightly with increasing concentration, but this was not of practical significance. Different batches of solid-phase cartridges did not affect the recovery.

Scrupulous drying of the columns before eluting was necessary, because of the elution with methanol. Small amounts of water catalysed the hydrolysis of penicillin-V by methanol during the evaporation. As a result of this reaction, the methylester of the penicilloic acid of penicillin-V was formed, which could be noticed as a broad peak with a retention time of about 6 min. Flushing with *tert.*-butylmethyl ether decreased the recovery slightly but helped in obtaining clear samples.

3.2. Stability studies

The stability of penicillin-V greatly depends on the pH. The rate of degradation is at its minimum between pH 6.0 and 6.5 [6], but it is also influenced by the type of buffer used.

To assess the stability of penicillin-V after sample preparation, blank plasma samples were extracted. After evaporation of the methanol, the residue was

Table 4
Precision and accuracy of the assay obtained during the analysis of study samples

Concentration (μg/ml)	Precision (%)	Accuracy (%)
0.536 (n=49)	7.5	−1.0
1.07 (n=51)	6.4	−0.9
2.57 (n=50)	8.2	−0.2
7.72 (n=53)	6.7	+0.9
15.4 (n=49)	5.5	+0.6

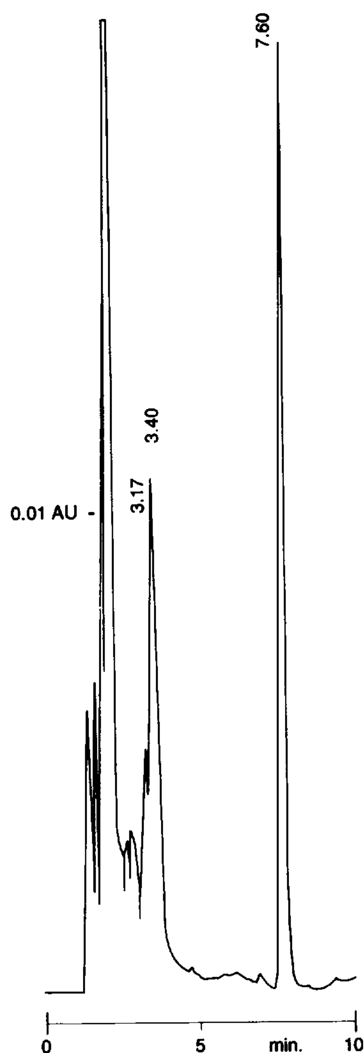

Fig. 3. Chromatogram of human plasma containing 5*R*- and 5*S*-penicilloic acids (3.17 and 3.40 min) and penicillin-V (7.60 min).

dissolved in citrate buffer containing 20 μg penicillin-V, K^+ per ml. The samples were stored in the auto-sampler at a temperature of 33°C and every hour a sample was injected. The stability of the penicillin-V in citrate buffer (0.003 *M*, pH 6.0) (reference solution) was also tested under the same conditions. Fig. 2 shows that the concentration in plasma samples decreased by 0.45%/h, while the reference solution decreased by only 0.11%/h. Clearly one of the extract constituents negatively influenced the stability of penicillin-V. Accepting a degradation of 5%, the samples could be stored for 11 h in the auto-sampler.

The stability of penicillin-V in plasma was investigated at −20°C and at room temperature. The results indicated that penicillin-V containing plasma samples could be stored for three months at −20°C without noticeable degradation. At room temperature samples should be analysed within 2 h.

3.3. Precision and accuracy

During validation, the precision and accuracy of the method were determined at three concentrations on two days (Table 2, Table 3). Both precision and accuracy appeared to be independent of concentration. More information on the precision and the accuracy was gathered during the analysis of study samples obtained from a bio-equivalence study. With each set of study samples, quality control samples at five concentrations between 0.54 and 15.4 μg/ml

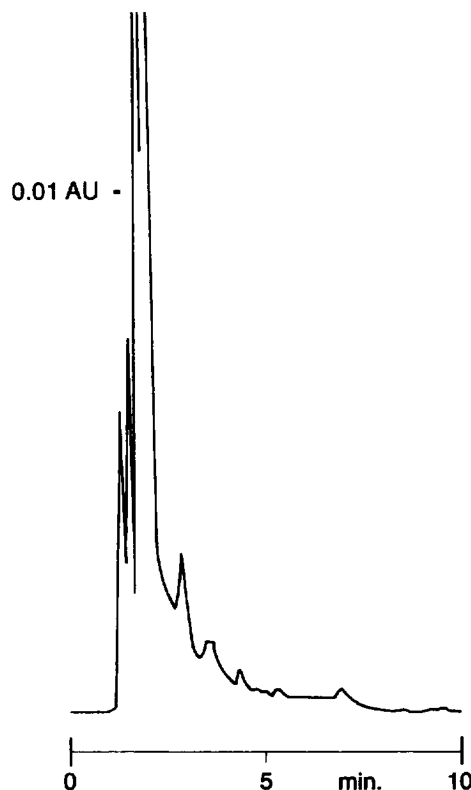

Fig. 4. Chromatogram of blank human plasma.

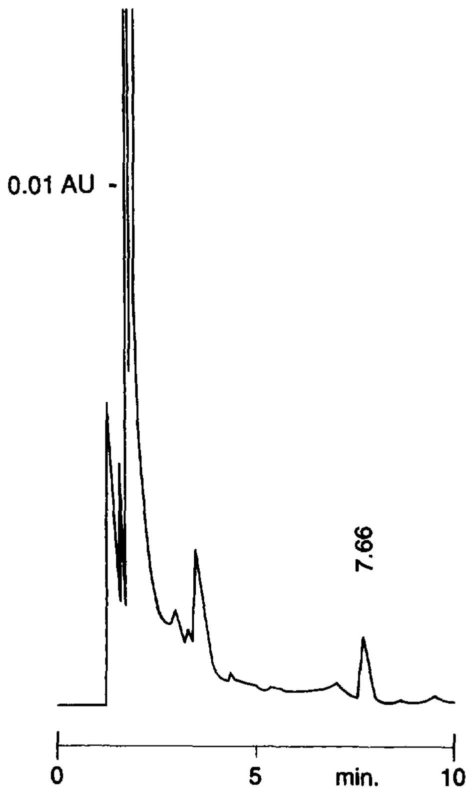

Fig. 5. Chromatogram of human plasma containing 0.3 μg penicillin-V/ml (7.66 min) obtained from a dosed volunteer.

were included to control the performance of the analysis. These results also proved that the method was precise and accurate (Table 4).

3.4. Reproducibility of the calibration graph

During the analysis of study samples, 19 calibration graphs were analysed over a period of two months. A mean slope of 41.9±2.4 and a mean intercept of 0.2±0.5 were calculated. The correlation coefficient was always higher than 0.9950 and in 14 cases higher than 0.999. These results showed that the calibration curve was reproducible over a period of two months.

3.5. Limit of quantification and limit of detection

The limit of quantification was defined as the lowest concentration that could be determined with an accuracy and a precision of at most 20%. It was calculated as the concentration giving a response equal to the blank, plus five times the standard deviation of the blank. The blank signal was determined by injecting 6 different batches of plasma and measuring the signal at the retention time of penicillin-V. At a concentration of 100 ng penicillin-V/ml plasma an accuracy of 13% and a precision of 12% was calculated.

The limit of detection was determined by interfering peaks and not by detector noise or pump noise. The limit of detection was therefore defined as the concentration which gave a peak height equal to the mean interference plus three times the standard deviation of the mean interference. To determine the mean interference, blank plasma samples of six different batches were analysed. A limit of detection of 35 ng/ml was found.

3.6. Linearity of the calibration curve

The suitability of the linear model for the calibration graph was tested by performing a lack-of-fit test [7]. All calibrators were prepared in duplicate. They were analysed in a random sequence to minimise time effects. The lack-of-fit test showed that there was no proof for a deviation of the calibration graph from linearity.

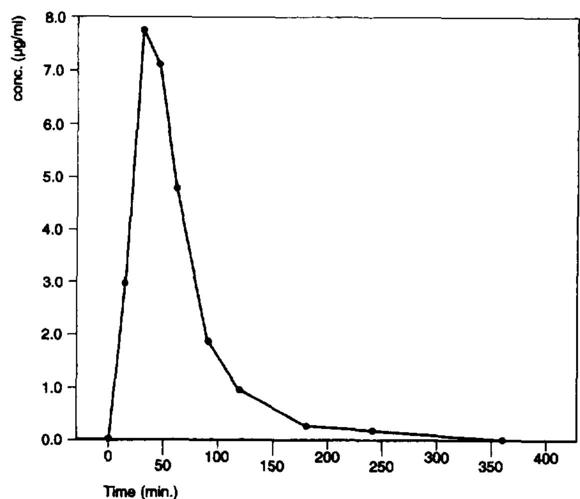

Fig. 6. Plasma concentration versus time curve after administration of 800 mg penicillin-V, K^+ to a volunteer.

3.7. Selectivity

Penicillin-V was well separated from its main metabolites, 5*R*- and 5*S*- penicilloic acid (retention times 3.2 and 3.4 min, Fig. 3). Although in most study samples obtained from dosed volunteers, the penicilloic acids were present, this assay was neither optimized nor validated for the determination of penicilloic acid.

To examine the possible interferences of endogenous components, six batches of blank plasma were extracted and analysed during validation. A mean interference of 13% at the lowest calibrator was found. During the analysis of study samples, 51 blank samples obtained from 20 volunteers were inspected for interferences. 37 samples showed no visible interferences, the remainder showed a mean interference of 14±5% compared to the lowest calibrator. Fig. 4 shows an example of a chromatogram of a blank plasma sample. Fig. 5 shows a chromatogram of a plasma sample containing 0.3 μg penicillin-V, K^+/ml obtained 2.5 h after administering 800 mg penicillin-V, K^+ to a volunteer.

4. Discussion

The method discussed has been used for the determination of penicillin V in human plasma for bio-equivalence studies. During the measurement of the samples, the method has proved to be reliable. An example of a plasma concentration versus time curve, after oral administration of 800 mg penicillin V, potassium salt to a volunteer, is shown in Fig. 6. A C_{max} of 7.8 μg/ml was reached at 30 min after administration, the area under the plasma concentration versus time curve was 495 μg min/h. Penicillin V was eliminated with a terminal half life of 30 min.

5. General remarks and conclusion

The validation and experience with the assay during the analysis of about 800 study samples have shown that the method described was reliable, selective, precise and accurate enough to determine penicillin-V between 0.1 and 19 μg/ml plasma. About 30–40 study samples could be analysed each day together with 8 calibrators and 6 quality control samples chosen from a set of quality control samples spiked at 5 different concentrations. In case more samples would have to be analysed on one day, the stability of the prepared samples should be increased and it is likely that the use of a cooled auto-sampler would be useful.

References

[1] M. Barza and L. Weinstein, Clin. Pharm., 1 (1976) 297.
[2] R.L.P. Lindberg, R.K. Huupponen and P. Huovinen, Antimicrob. Agents Chemother., 26 (1984) 300.
[3] E. Mendez-Alvarez, R. Soto-Otero, G. Sierra-Paredes, E. Aguilar-Veiga, J. Galan-Valiente and G. Sierra-Marcuño, Biomed. Chromatogr., 5 (1991) 78.
[4] W.A. Moats, J. Chromatogr., 593 (1992) 15.
[5] J.O. Boison, G.O. Korsrud, J.D. MacNeil and L. Keng, J. Chromatogr., 576 (1992) 315.
[6] H. Bundgaard and C. Larsen, Int. J. Pharm., 1 (1978) 95.
[7] S.N. Deming and S.L. Morgan, Clin. Chem., 25 (1979) 840.