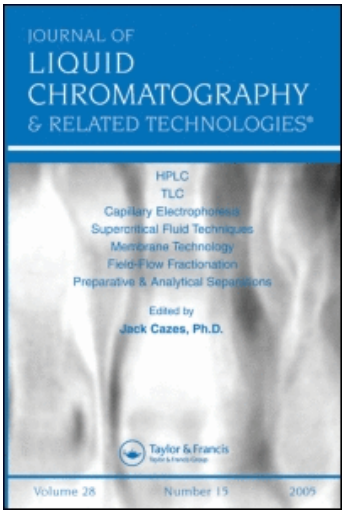

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETECTION OF RESIDUAL FORMALDEHYDE IN A HEPATITIS-A VACCINE BY USE OF HYDRALAZINE

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ABSTRACT

An assay was developed that is capable of detecting levels of residual formaldehyde in viral vaccine dosage formulations to 0.01 µg/mL. The assay employs incubation of dosage formulation suspensions with hydralazine hydrochloride under mildly acidic conditions and elevated temperatures. Formaldehyde present is derivitized to yield s-triazolo-[3,4-a]-phthalazine. Selectivity of the assay is enhanced by performing reverse phase HPLC after the reaction, and monitoring elution of the fluorescent product. The presence of insoluble aluminum hydroxide in dosage formulations does not affect formaldehyde recovery. The assay was used to determine formaldehyde levels in dosage formulations and in in-process samples of a newly developed Hepatitis A vaccine.

INTRODUCTION

Protection against viral diseases is afforded by protective antibodies induced by innoculation with specific viral antigens. These antigens have included low virulent wild type virus at low doses, inactivated viruses, attenuated viruses, or viral fragments produced by either disruption or recombinant techniques (1).

If the viral antigen is viable, it is imperative to thoroughly inactivate under stringent conditions. Virus inactivation can be accomplished by a number of procedures, including heat, UV irradiation, and exposure to beta-propiolactone, formaldehyde, ozone, chlorine, phenol, sodium hypochlorite, or detergents (2-7). The inactivation procedure chosen for a particular virus has to be chosen empirically, because some viruses are resistant to selected inactivating agents.

Recently, a vaccine against Hepatitis A has been developed (8). This vaccine utilizes an attenuated Hepatitis A viral strain grown in cultured cells and purified to high protein purity. The attenuated virus is inactivated by incubation with a formaldehyde solution. Since formaldehyde is a known toxin at low levels (9), removal of the compound is of importance. It is then necessary to assay the vaccine dosage formulation to confirm removal of formaldehyde.

Many different procedures have been employed over the past several decades to quantitate residual formaldehyde in aqueous solutions. Due to the low molar absorbance of formaldehyde, most analytical procedures have utilized derivatization of formaldehyde

with spectrophotometric analysis of the reaction products. Common derivatization reagents include 2,4-pentanedione (10), phenylhydrazine (11), 2,4-dinitophenylhydrazone (12), chromotropic acid (13), 3-methyl-2-benzothiazolone (14), and tryptophan (15). Since many of these reagents may react with other aldehydes that may be in the same sample matrix, it is possible to enhance selectivity of the assay by separating the reaction products by either liquid or gas chromatography.

Initial attempts to quantify residual formaldehyde in the Hepatitis-A vaccine dosage formulation used phenylhydrazine as the derivatizing reagent. Formaldehyde levels of all lots were below the limit of detection of the assay, which was 1.6 ppm (16). It was desired to develop a more sensitive method to provide absolute quantitation, and to enable confirmation of manufacturing consistency by comparing residual formaldehyde levels in different lots. A new, more sensitive, method was developed which consists of derivatization of the vaccine dosage formulation with hydralazine hydrochloride, followed by centrifugation, addition of acetonitrile, and reverse phase high performance liquid chromatography (HPLC). Elution of the derivative, s-triazolo-[3,4-a]-pthalazine (Tri-P) is monitored with a fluorescence detector. Parameters of the assay were determined, and are presented in this report.

MATERIALS AND METHODS

Chemicals

Hydralazine hydrochloride was purchased from Aldrich Chemical (Milwaukee, WI). Optima grade acetonitrile and HPLC grade

potassium phosphate monobasic were purchased from Fisher Scientific (Pittsburgh, PA). Sequanal grade trifluoroacetic acid was purchased from Pierce Chemicals (Rockford IL). Other chemicals were either certified or reagent grade. Chemicals were used as purchased, without further purification. Solutions and solvents were made with high quality water (>14 MΩ) obtained from a Milli-Q unit (Millipore Corporation, Waltham MA). All HPLC solvents were passed through a 0.45 micron filter and degassed prior to use.

Standard and Sample Preparation

A 50 mcL aliquot of 36.6% (w/w) aqueous formaldehyde was diluted with water to 1 L to create a 20 µg/mL solution of formaldehyde. Subsequent dilutions were made to create standard solutions ranging from 0.005 to 1.0 µg/mL. New pipette tips were used for each dilution transfer to prevent carryover of formaldehyde.

One mL of standard solution was added to 1 mL of derivatization reagent (0.1 mg/mL of hydralazine hydrochloride in 0.2 M potassium phosphate monobasic). The mixture was vortexed and then incubated for 15 min at 100C. After incubation, the mixture was centrifuged at 900 *g* for 4 min at 5C in a Sorvall model RT-6000D centrifuge (DuPont Biotechnology Systems, Wilmington, DE). One mL of acetonitrile was then added, and the mixture vortexed prior to placement into an autosampler vial.

Two sets of blanks were prepared in parallel to the standards. One set contained one mL of water and 1 mL of the derivatization

reagent, while the second contained 2 mL of water. Both sets were then incubated for 15 min at 100C, centrifuged as described above, and added to 1 mL of acetonitrile.

Sample solutions were derived from the Hepatitis-A vaccine formulation, which is a suspension of the inactivated and purified attenuated virus adsorbed onto aluminum hydroxide. A sample solution was treated in one of three manners: [1] The contents of a vaccine dosage vial were centrifuged at 900 *g* for 4 min at 5C in a Sorvall model RT-6000D centrifuge (DuPont Biotechnology Systems, Wilmington, DE), and 0.4 mL of supernatant carefully removed. The 0.4 mL supernatant aliquot was added to 0.4 mL of derivatization solution, and treated as the standards, adding 0.4 mL acetonitrile instead of 1 mL. [2] Fifty µL of 10% trifluoroacetic acid (v/v) was added to 0.45 mL of vaccine suspension and the mixture vortexed. Visual observation revealed that the aluminum hydroxide went into solution 5 min after addition of acid. The 0.5 mL acidified vaccine suspension was added to 0.5 mL of derivatization solution, and treated as the standards, adding 0.5 mL acetonitrile instead of 1 mL. [3] 0.5 mL of vaccine suspension was added to 0.5 mL of derivatization solution, and incubated as the standards. After incubation, the mixture was centrifuged as described above, and a 0.8 mL aliquot of supernatant removed and added to 0.4 mL of acetonitrile.

All manipulations were performed in glass vessels. All standard, blank and sample preparations were performed in duplicate.

Chromatographic Conditions

The HPLC system consisted of two model 510 pumps controlled by a model 680 automated gradient controller (Waters Associates, Millipore Corporation, Waltham, MA), a model AS4000 refrigerated autosampler set at 5C (Hitachi Instruments, Danbury CT), and a model LC240 fluorescence detector (Perkin-Elmer, Norwalk, CT) . Injection volume was standardized at 20 µL with a 100 µL sample loop. Separation was performed on a C_{18} reverse phase HPLC column (4.6 mm ID x 15 cm length, Vydac-The Separations Group, Hesperia, CA) using a linear gradient of 70:30 to 57:43 of 50 mM potassium phosphate:acetonitrile over 10 minutes at a flow rate of 1 mL/min. The fluorescence emission of the column eluent was monitored at 398 nm with excitation set at 239 nm. Data were collected and analyzed with a PE Nelson Access* Chrom chromatography data acquisition system (Cupertino, CA).

RESULTS

Optimization of the Derivatization Reaction

Initial experiments were performed to determine derivatization conditions which yield maximal fluorescence. Parameters examined included concentration of hydralazine hydrochloride, and the length and temperature of incubation. Optimal derivatization conditions were determined to be 15 minutes at 100C with a reagent solution containing 0.1 mg hydralazine hydrochloride/mL (Tables 1 and 2). Under these conditions the sample volume remained constant.

TABLE 1

Impact of Incubation Temperature and Duration on Peak Response. (RT = ambient room temperature)

Temp. - Duration	Formaldehyde Concentration	
	0.01 µg/mL % maximum	0.4 µg/mL % maximum
RT - 15 min	78%	19%
RT - 30 min	68%	20%
50C - 15 min	70%	33%
50C - 30 min	70%	42%
80C - 15 min	100%	93%
80C - 30 min	86%	96%
100C - 15 min	99%	100%

TABLE 2

Impact of Hydralazine•HCl Concentration on Peak Response with Incubation at 100C for 15 min.

Hydralazine•HCl	Formaldehyde Concentration	
	0.01 µg/mL % maximum	0.4 µg/mL % maximum
0.01 mg/mL	70%	69%
0.1 mg/mL	100%	99%
0.5 mg/mL	54%	100%

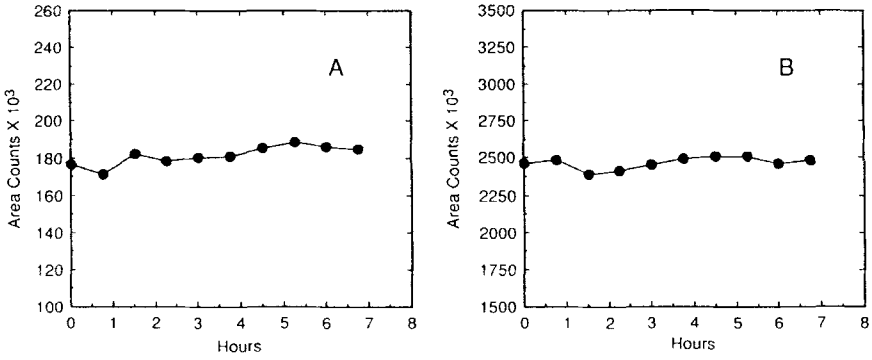

FIGURE 1. Stability of the fluorescent derivative, Tri-P. Ten sequential injections were made from the same sample vial at 45 min intervals. [A] Sample vial which contained 0.01 µg/mL formaldehyde, [B] Sample vial which contained 0.5 µg/mL formaldehyde.

Multiple injections from two derivatized samples at different formaldehyde concentrations revealed no loss of fluorescence over 6.5 hours (Figure 1).

Linearity and Sensitivity

Formaldehyde concentration was found to be directly proportional to peak area over at least two orders of magnitude, from 0.01 µg/mL to 1.0 µg/mL formaldehyde (r^2=0.9995). A decline in linearity, noted above 1.0 µg/mL, was likely due to overload of the detector by the high concentration. The limit of detection was 0.005 µg/mL (at 10X S/N) and the limit of quantitation was 0.01 µg/mL.

Precision

Method precision of this assay was measured by the extent from which individual data points of the standard curve deviated from the

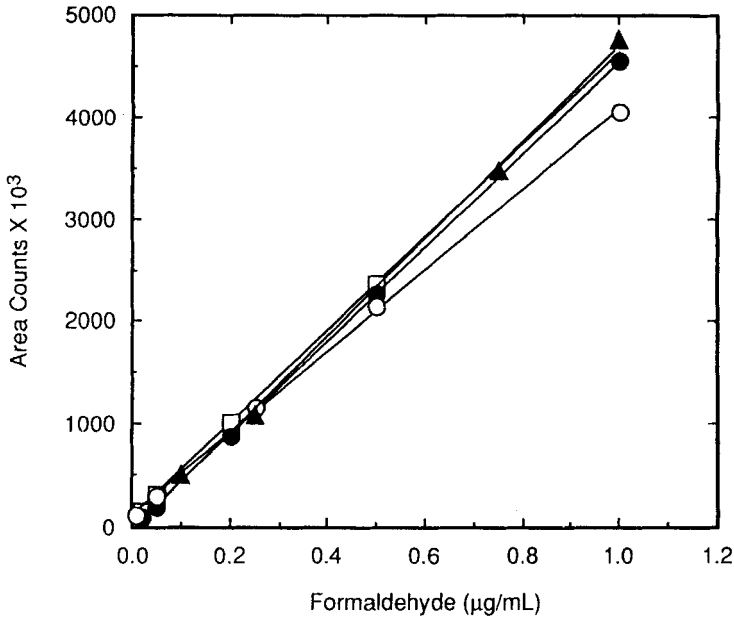

FIGURE 2. Method precision: Deviation of data points from the linear regressions. Four different standard curves (each represented with a different symbol) were evaluated. Standard curves were run on different days.

value predicted from the linear regression. Four standard curves were evaluated in this manner (Figure 2). The values obtained from 0.01 µg/mL to 1.0 µg/mL ranged from 86% to 127% of that predicted by the linear regression, with typical values between 95% to 105% (Table 3). The system precision of the assay was determined by making multiple injections from a set of standard solutions with known formaldehyde contents. System precision, using the equipment described here, was 2.8% RSD at 0.01 µg/mL (n=10), and was 1.6% RSD at 0.5 µg/mL (n=10).

TABLE 3

Method Precision, Measured by the Extent from which Individual Data Points from Four Standard Curves Deviated from the Values Predicted from the Linear Regressions.

	Experimental/Theoretical x 100			
Formaldehyde Concentration	standard curve #1 (r^2=0.9999)	standard curve #2 (r^2=0.9998)	standard curve #3 (r^2=0.9995)	standard curve #4 (r^2=0.9997)
0.01 µg/mL				86%
0.02 µg/mL	127%	97%		
0.05 µg/mL	99%	102%		101%
0.1 µg/mL			115%	
0.2 µg/mL	98%	103%		
0.25 µg/mL			95%	105%
0.5 µg/mL	100%	99%	100%	103%
0.75 µg/mL			98%	
1.0 µg/mL	99%		101%	99%

TABLE 4

Influence of Extraction Procedure on Formaldehyde Recovery.

Extraction Procedure	Formaldehyde Recovered
Centrifugation, assay of supernatant.	0.27 µg/mL
Acid dissolution of suspension	0.34 µg/mL
Direct assay of suspension.	0.42 µg/mL

Recovery from Dosage Formulation

Experiments were performed to examine the possibility that the aluminum hydroxide used to adsorb the viral antigen in the Hepatitis A vaccine dosage formulation (8,16) might interfere with the recovery of the formaldehyde in dosage vials. Three different procedures were evaluated to maximize formaldehyde recovery from the actual dosage vials: [1] Centrifugation of the dosage formulation and assay of the supernatant, [2] Dissolution of the aluminum hydroxide with acid, and [3] Direct assay of the suspension. Highest formaldehyde recovery was obtained with the direct assay of the suspension (Table 4). Complete recovery of formaldehyde was confirmed in experiments in which a suspension of aluminum hydroxide identical to that used in the dosage vials was spiked with either 0.02 µg/mL or 0.5 µg/mL formaldehyde. Recoveries were 98.0% (0.02 µg/mL) and 99.4% (0.5 µg/mL).

TABLE 5

Residual Formaldehyde in Doses of Different Lots of Hepatitis A Vaccine.

	Formaldehyde Content (μg/mL)
Lot 001	0.42
Lot 002	0.69
Lot 003	0.34
Lot 004	0.89
Lot 005	0.35
Lot 006	0.36

A practical measure of assay reproducibility was obtained by assaying a designated lot of vaccine dosage formulation on three different occasions, utilizing freshly generated standard curves. The results obtained were 0.33 μg/mL, 0.34 μg/mL, and 0.34 μg/mL.

Assay of Samples

Six lots of vaccine dosage formulation were assayed for the presence of residual formaldehyde using the phenylhydrazine procedure and the procedure described in this report. The phenylhydrazine procedure revealed that each lot contained $\leq$ 1.6 μg/mL formaldehyde (16). The actual levels of formaldehyde were found to range between 0.34 μg/mL and 0.89 μg/mL (Table 5). The relatively close values obtained for the different lots confirms consistency of manufacture. Elution of the Tri-P peak at 2.7 min (Figure 3) was consistent for all lots and standards examined (data not shown).

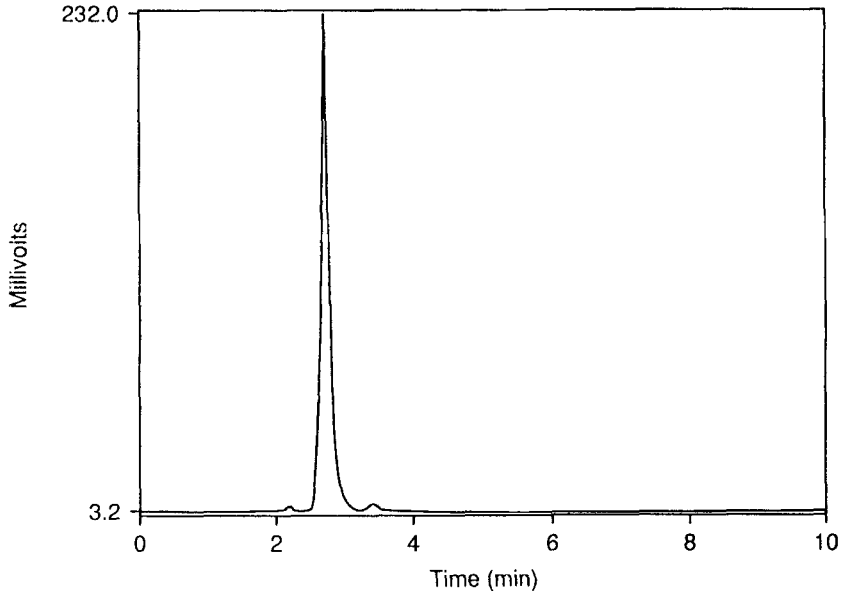

FIGURE 3. Fluorescence chromatogram of a representative lot of Hepatitis A vaccine formulation, after derivitization with hydralazine hydrochloride. The Tri-P peak elutes at 2.7 minutes. HPLC conditions are described in the text.

TABLE 6

Formaldehyde levels in Sequential Supernatant Fractions from the Isolation Procedure of a Representative Lot.

	Theoretical (µg/mL)	Experimental (µg/mL)
supernatant #1	100	96
supernatant #2	40	48
supernatant #3	15	19.2
supernatant #4	5	7.2

A set of in-process samples from sequential steps in the purification procedure were analyzed for residual formaldehyde. The results are compared to the theoretical, based on the dilutions inherent in the isolation protocol (Table 6). The experimental values are in close agreement with the theoretical, and confirm the removal of formaldehyde from the Hepatitis A vaccine dosage formulation.

DISCUSSION

Formaldehyde inactivation has been demonstrated for a wide variety of viruses, including poliovirus, influenza, mumps, respiratory syncytial virus, Hepatitis A, Hepatitis B, and the human immunodeficiency virus (1-4, 17). In general, when using inactivated virus for the manufacture of vaccines, it is common to extend the inactivation steps to provide assurance that all virus present has been inactivated. Provost et al. (4) demonstrated that the Hepatitis-A virus could be completely inactivated by exposure to formaldehyde at a 1:4000 dilution for 72 hours at 37C. The virus inactivation procedure used in the manufacture of the newly developed vaccine consisted of a 20 day exposure at 37C to 37% (w/w) formaldehyde at a dilution of 1:4000, giving a concentration of 100 μg/mL (16). After exposure to formaldehyde, the bulk virus is adsorbed to aluminum hydroxide, and subjected to a series of sedimentations and resuspensions which reduce the level of formaldehyde (16). On a theoretical basis, the formaldehyde content of the vaccine dosage formulation should be <2 μg/mL.

Numerous protocols have been used to detect residual formaldehyde in viral vaccines. In a comparative study (18), it was

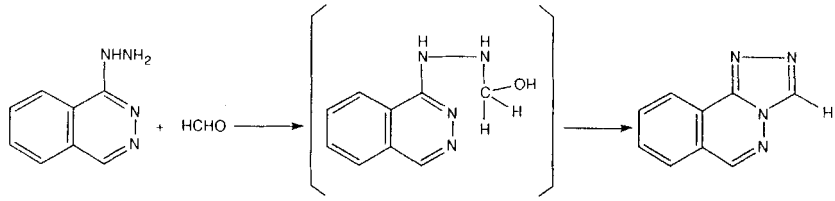

FIGURE 4. Reaction of hydralazine hydrochloride with formaldehyde to yield s-triazolo-[3,4-a]-phthalazine [Tri-P], (19).

demonstrated that protocols using the derivitizing reagents 2,4-pentanedione, phenylhydrazine, chromotropic acid, 3-methyl-2-benzothiazolone, and tryptophan were equivalent in accuracy. The formaldehyde content of the five vaccines examined in that study ranged from 55 µg/mL to 1526 µg/mL. Once accuracy and sensitivity are determined not to be limiting, the choice of protocol is determined by assay precision. The coefficient of variation of the different procedures (18) was lowest when 2,4-pentanedione was the derivitizing reagent, and was highest with phenylhydrazine. However, at low levels, the coefficient of variation of the different procedures was equivalent. The protocol using phenylhydrazine as a derivitizing reagent has an advantage at very low levels because sensitivity can be extended to 1.6 µg/mL. Using phenylhydrazine as the derivitizing reagent, manufactured lots of the newly developed vaccine were demonstrated to contain<1.6 µg/mL of formaldehyde (16). While satisfactory in providing assurance of the safety of the product, a more sensitive method was desired to monitor lot to lot consistency.

Noda et al. (19) reported that hydralazine hydrochloride could be reacted with formaldehyde to form a fluorescent compound, s-triazolo-[3,4-a]-phthalazine (Tri-P) (Figure 4). Tri-P can be excited at 239 nm, and the emission monitored at 398 nm. In contrast, hydralazine hydrochloride does not fluoresce under those conditions. Tri-P can be quantitated directly either spectrophotometrically or by HPLC methods. This reaction was adapted to quantitate residual formaldehyde in the attenuated, formaldehyde inactivated, Hepatitis-A vaccine recently developed, as well as in in-process samples from the manufacturing procedure. The formaldehyde contents of all dosage lots tested were below the limit of detection of the prior assay procedure, but could be readily quantified by the method described here.

CONCLUSIONS

The assay developed employs only one reaction step and a subsequent solvent addition prior to injection into an HPLC. We have demonstrated that the procedure is simple, sensitive, accurate, precise, and rugged, making it practical for routine use for assay of viral vaccines. The simplicity and selectivity of this procedure also make it likely that this assay can also be utilized to detect formaldehyde in a wide variety of other matrices, including pharmaceutical and environmental samples.

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REFERENCES

1. E. Norrby, "Strategy for virus vaccine development", in Applied Virology, E. Kurstak, ed., Academic Press, Orlando FL, 1984, pp 3-16.

2. E.J. Morris, and H.M. Darlow, "Inactivation of viruses", in Inhibition and Destruction of the Microbial Cell, W.B. Hugo, Academic Press, London, 1971, pp 687-702

3. B. Spire, F. Barre-Sinoussi, L. Montagnier, J.C. Chermann, Lancet, ii: 899-901, 1984.

4. P.J. Provost, B.S. Wolanski, W.H. Miller, O.L. Ittensohn, W.J. McAleer, and M.R. Hilleman, Proc. Soc. Exp. Biol. Med., 148: 532-539, 1975.

5. G.G Frosner, W. Stephan, and H. Dichtmuller, Eur. J. Clin. Microbiol. 2: 355-357, 1983.

6. K. Herbold, B. Flehmig, and K. Botzenhart, Appl. Environ. Microbiol. 55: 2949-2953, 1989.

7. W.O. Grabow, V. Gauss-Muller, O.W. Prozesky, and F. Deinhart, Appl. Environ. Microbiol. 46: 619-624, 1983.

8. A. Werzberger, B. Mensch, B. Kuter, L. Brown, J. Lewis, R. Sitrin, W. Miller, D. Shouval, B. Weins, G. Calandra, J. Ryan, P. Provost, and D. Nalin, New England J. Med. 327: 453-457, 1992.

9. M.O. Amdur, J. Doull, and C.D. Klaassen, Casarett and Doull's Toxicology: The Basic Science of Poisons, McGraw Hill, New York, NY 1991.

10. T. Nash, Biochem. J., 55: 416-421, 1953.

11. M. Tanenbaum, and C.E. Bricker, Anal. Chem. 23: 354-357, 1951.

12. B.A. Tomkins, J.M. McMahon, W.M. Caldwell, and D.L. Wilson, J. Assoc. Off. Anal. Chem., 72: 835-839, 1989.

13. E. Sawicki, T.R. Hauser, and S. McPherson, Anal. Chem. 34: 1460-1464, 1962.

14. E. Sawicki, R.T. Hauser, T.R. Stanley, and W.C. Ebert, Anal. Chem. 33: 93-96, 1961.

15. J. Chrastil, and J.T. Wilson, Anal. Biochem, 63: 202-207, 1975.

16. J.A. Lewis, M.E. Armstrong, V.M. Larson, E.A. Emini, K. Midthun, E. Ellerbeck, D. Nalin, P.J. Provost, and G.B. Calandra, "Use of a live attenuated Hepatitis A vaccine to prepare a highly purified formalin inactivated Hepatitis A vaccine", in Proceedings of the 1990 International Symposium on Viral Hepatitis and Liver Disease: Contemporary Issues and Future Prospects, F.B. Hollinger, S.M. Lemon, and H. Margolis, eds., Williams and Wilkins, Baltimore MD, 1991, pp 94-97.

17. F. Deinhardt, W. Jilg, R. Zachoval, J. Abb, M. Roggendorf, and G. Zoulek, "Vaccination of newborns against Hepatitis B", in Applied Virology, Kurstak, E., ed., Academic Press, Orlando FL, 1984, pp 191-197.

18. G.N. Frerichs, and M.D. Chandler, J. Biol. Stand, 8: 139-144, 1980.

19. H. Noda, M. Minemoto, A. Noda, and U. Toshihiko, Chem. Pharm. Bull. 34: 3499-3501, 1986.