Pergamon

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 6, pp. 833–837, 1994

0731–7085/94 $7.00 + 0.00

0731–7085(94)E0004-K

Reversed-phase LC assay method for deoxycholate in influenza vaccine

MICHEL GIRARD,*† KIM HANNAH,‡ J. ALLAN MENZIES,† GNANAPRAKASAM SOLOMONRAJ† and LARRY W. WHITEHOUSE†

†*Bureau of Drug Research, Health Canada, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2*
‡*Bureau of Biologics, Health Canada, Canada*

Abstract: Sodium deoxycholate is used for the disruption of particles in the manufacturing of some influenza vaccines. Residual deoxycholate in inactivated vaccines is currently determined using a labour-intensive colorimetric method which lacks complete specificity. An alternative assay method for residual deoxycholate in vaccine preparations was developed using reversed-phase LC. Cholic acid was used as internal standard and the ratio of internal standard to test solute was used for all calculations. Prior to LC analysis, deoxycholic acid was concentrated by solid-phase extraction, a procedure that also removed proteinaceous material in vaccine samples. The clean-up/concentration procedure recovery was examined using untreated samples and was found to be quantitative. The linearity range of the LC method was between 3 and 200 μg ml^{-1}, with a limit of detection of approximately 0.4 μg on column, and a lower limit of quantitation of 1.6 μg on column. Replicate assays during intra- and inter-day experiments gave acceptable levels of variability. The DCA content of samples from three lots of influenza vaccine varied between 10 and 16 μg ml^{-1}. These values were appreciably lower than those measured spectrophotometrically, indicating the higher specificity of the LC method.

Keywords: *Deoxycholate; HPLC; influenza vaccine; quantitation; assay.*

Introduction

Influenza virus particles can be successfully disrupted by means of biological or chemical approaches [1–3]. In particular, the development of detergent split, inactivated influenza virus vaccines followed the report of animal protection studies by Webster and Laver [4] on vaccines disrupted with sodium deoxycholate. Subsequent vaccine trials in human volunteers demonstrated that the deoxycholate split vaccine conferred a significant degree of protection against infection by influenza virus [5–7].

The development of improved influenza vaccines of high immunogenicity and low reactogenicity is important in increasing the successful use of inactivated vaccine. In deoxycholate split vaccines, the detergent concentration can be reduced following processing of bulk material by dialysis or filtration. Residual traces of deoxycholate detergent, which is present naturally in the body, are not thought to be harmful [8]. However, quantitation of excipients in vaccine products is required to minimize the potential risk of toxicity to humans. At present, residual deoxycholate is determined by a labour-intensive colorimetric method [9] which lacks specificity, and from which deoxycholate levels present are calculated by extrapolation.

Several LC methods have been described for the analysis and quantitation of bile acids and their conjugates in biological fluids, and they have been the subject of a review [10]. Because of the relatively low abundance of the free acids, most methods have been concerned with conjugates and often necessitated fractionation, concentration and derivatization steps prior to analysis. Free bile acids such as ursodeoxycholic and chenodeoxycholic acids are used in pharmaceutical preparations and have been assayed directly by LC without preconcentration or derivatization due to the relatively large amounts present (200–400 mg $dose^{-1}$) [11].

This study was undertaken to develop a highly sensitive and specific LC assay method for the detection and quantitation of deoxycholic acid (DCA) in final product prep-

* Author to whom correspondence should be addressed.

arations of split, inactivated influenza vaccine and to compare it to a currently used colorimetric method.

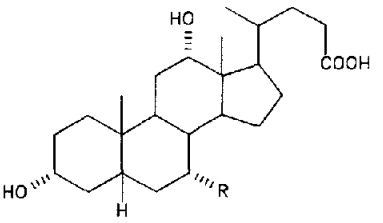
Experimental

Materials

Deoxycholic acid (DCA), (Lot 50H0001) and cholic acid (CA), (Lot 128f0819) were supplied by Sigma (Toronto, Canada). Analytical grade sodium acetate was purchased from BDH (Toronto, Canada) and HPLC-grade methanol and acetonitrile were supplied by Johns Scientific (Toronto, Canada). All other chemicals were of analytical grade and purchased from local suppliers. Milli-Q water ($18M\Omega$ cm^{-1}, 0.22 μm filtered) was used to prepare all solutions. Ampoules from three lots of DCA-containing vaccine and blank vaccine were supplied by the Bureau of Biologics (Health Canada) for assessment. Each ampoule contained 6 ml of vaccine preparation.

Liquid chromatography

Chromatographic separations were performed isocratically at room temperature on a Spectra-Physics SP8100 liquid chromatograph equipped with a Spectroflow 783 programmable detector (ABI Analytical, Toronto, Canada) and a Spectra-Physics SP4400 Integrator. The detector was set at 210 nm with a sensitivity of 0.02 a.u.f.s. Samples were analysed by reversed-phase LC on a 5 μm Supelcosil LC-18-DB column (250 mm × 4.6 mm i.d.) (Supelco Canada, Oakville, Canada), using a pre-mixed mobile phase of methanol–acetonitrile–0.02 M sodium acetate (37.5:37.5:25), the latter adjusted to pH 4.3 with 1 M phosphoric acid. The mobile phase was degassed and filtered through a 0.2 μm filter (Ultipor Nylon66) prior to chromatography and elution was carried out at a flow rate of 1.0 ml min^{-1}.



Cholic acid R=OH

Deoxycholic acid R=H

Stock solutions

Stock solutions of DCA and CA were prepared in methanol, each at a nominal concentration of 2.5% (w/v). The CA stock solution was used without further dilution as internal standard.

Vaccine vehicle

Phosphate buffered saline solution, 0.15 M ($Na_2HPO_4 \cdot 7H_2O$, 1.67 g l^{-1} and KH_2PO_4, 0.2 g l^{-1}) pH 7.3, containing 8.0 g l^{-1} and 0.2 g l^{-1}, respectively, of sodium chloride and potassium chloride was used as vaccine vehicle.

Standard solutions

DCA standard solutions were prepared in methanol at concentrations of 0.39, 0.78, 1.56, 3.13, 6.25, 12.50 and 25.0 mg ml^{-1} using the appropriate dilution from the DCA stock solution.

Assay solutions

Standard assay solutions were prepared by transferring aliquots (2.5 ml) of vehicle or blank vaccine (vaccine material containing no DCA) to test tubes, each containing 20 μl of one of the DCA standard solutions and 20 μl of CA stock solution. The final DCA concentration ranged from 3.13 to 200 μg ml^{-1}. The pH of the solutions was adjusted by addition of 70 μl of 1 M phosphoric acid with vortexing. Similarly, sample assay solutions were prepared from aliquots (2.5 ml) of DCA-containing vaccine, spiked with 20 μl of internal standard and the pH was adjusted with phosphoric acid.

Assay method

Each standard and sample assay solution was passed through a C_{18}, 3 ml disposable Baker-10SPE (J.T. Baker, Phillipsburg, NJ, USA) cartridge, pre-washed with methanol and water, discarding the eluant and recovering the DCA by elution with methanol (2.5 ml). After removal of methanol under nitrogen at 40°C, the residue was dissolved in 0.2 ml of mobile phase and, using a 1 ml glass syringe, filtered through an Acro LC3S, 0.45 μm syringe filter (Gelman Sciences, Montreal, Quebec,

Canada). A 20 μl aliquot was assayed by LC for DCA.

Data analysis

All assays were performed in duplicate and results are expressed as mean ± SEM (standard error from the mean), unless otherwise indicated. Standard curves were determined by least-square regression from the log–log transformed data.

Results and Discussion

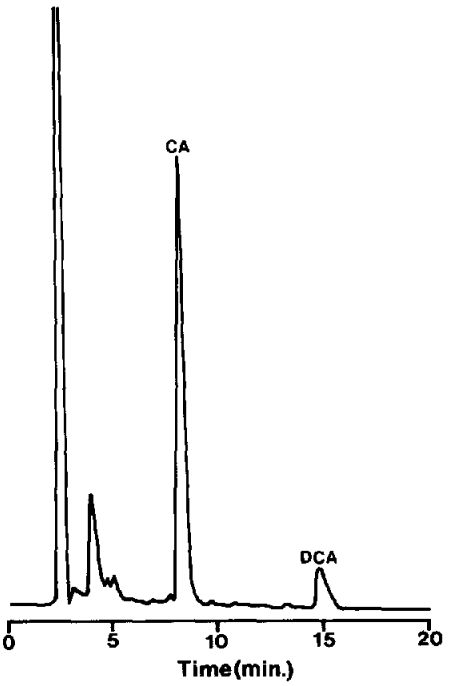
Extraction and recovery

Detection of free bile acids using direct UV monitoring at 210 nm has been used in published LC methods [10–12] with reported limits of detection of approximately 0.4 μg on column [10]. Since DCA levels of less than 50 μg ml^{-1} (less than 1 μg for a 20 μl injection) are expected in the split influenza vaccines, clean-up and concentration steps were required for adequate detection. Previous reports have shown that the use of small C_{18}-bonded silica cartridges provide easy clean-up and removal of interfering substances as well as giving excellent recoveries [11]. Thus, sample and standard assay solutions were passed through 3-ml disposable Baker-10SPE cartridges and the retained DCA was recovered by elution with methanol. The residue obtained after removal of methanol was reconstituted in a small amount of mobile phase corresponding to greater than 10-fold concentration, and subsequently analysed by LC. Both DCA and CA were recovered quantitatively by this process as demonstrated by the similar detector responses obtained for treated and untreated samples (data not shown). In the latter experiment, untreated samples corresponded to assay solutions prepared in mobile phase that had not been subjected to the clean-up procedure. Assay solutions prepared 24 h prior to analysis showed no apparent degradation in their DCA and CA content.

Chromatography

Chromatographic conditions were developed through a systematic analysis of several parameters that included column efficiency and selectivity, precision, reproducibility, linearity and sensitivity. Using octadecylsilyl-bonded silica columns, elution conditions were developed, starting with those reported by Scalia *et al.* [12], and using standard solutions of DCA and CA, the latter chosen as internal standard. Optimized separation conditions were obtained on a Supelcosil-LC-18-DB and isocratic elution with a mobile phase consisting of methanol–acetonitrile–0.02 M sodium acetate, pH 4.3 (37.5:37.5:25). Figure 1 presents a chromatogram showing the separation of the two standard acids, CA and DCA (50 μg and 6.3 μg injected, respectively), and calculated values for capacity factor (k'), asymmetry factor, plate count and resolution factor.

Linearity and range. Standard assay solutions ranging from 3.13 to 200 μg ml^{-1}, a range covering 30–400% of the expected range of residual DCA in vaccines (estimated at between 10 and 50 μg ml^{-1}) were prepared and assayed in triplicate according to the procedure described in the experimental



Test solute	k'	Asymmetry factor	Plate count*	Resolution factor
CA	3.0	1.2	8900	—
DCA	7.5	1.4	16500	8.75

* Expressed as the number of plates per metre.

Figure 1
RP-HPLC chromatogram of a mixture of deoxycholic acid (DCA) (6.3 μg) and cholic acid (CA) (50 μg) on a Supelcosil-LC-18-DB, 250 mm × 4.6 mm i.d., using an isocratic elution of methanol–acetonitrile–0.02 M sodium acetate, pH 4.3 (37.5:37.5:25), at a flow rate of 1 ml min^{-1}.

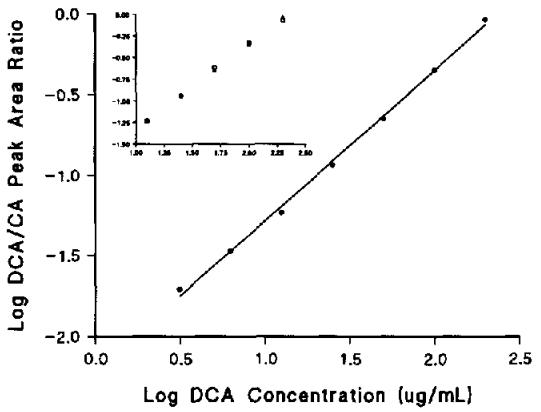


Figure 2
Log–log plot of deoxycholic acid (DCA) standard curve. Inset: Log–log plots of DCA standard curve with (Δ) and without (□) vaccine matrix.

section. A log–log plot of the average DCA to CA peak area ratio versus DCA concentration (Fig. 2) indicated that the method was linear over the full range of concentrations assayed, with the coefficient of determination (R^2) of 0.9985. The slope and intercept were 0.97 and −2.29, respectively. In a related experiment the effect of the vaccine matrix on the linearity was evaluated by assaying standard solutions prepared with blank vaccine solution. The log–log plot (Fig. 2 inset) was virtually identical with the standard curve prepared from non-vaccine material, thereby indicating that no loss of DCA or CA occurs in the presence of vaccine material.

Limit of detection and lower limit of quantitation. The limit of detection and the lower limit of quantitation were determined by assessing the response of replicate assays of standard solutions prepared at progressively lower concentrations. The limit of detection was attained at approximately 0.4 μg DCA on column (corresponding to assay of standard solution at 1.56 μg ml^{-1}) where the signal-to-noise ratio was about 3:1. The lower limit of quantitation, calculated at the concentration for which the coefficient of variation for six replicate injections was approximately 10%, was found to be 1.6 μg DCA on column, corresponding to assay of the 6.25 μg ml^{-1} standard solution.

Precision and reproducibility. The precision of the method was evaluated by examining intra- and inter-day variations of the peak response for replicate assays of the 25 μg ml^{-1} DCA standard assay solution (Table 1). Values for the DCA/CA peak area ratio demonstrated low variability for both intra- and inter-day experiments, with relative standard deviations of 3 and 4%, respectively.

Analysis of vaccine samples

Vaccine samples from three lots of a single manufacturer were analysed by LC and by the currently used colorimetric method, the latter based on the published article by Szalkowski and Mader. Standard curves for both methods were obtained starting from the same standard solutions. The colorimetric method was found to be linear for concentrations between 25 and 200 μg ml^{-1}. The results are summarized in Table 2.

The DCA levels determined by LC for vaccine lots nos 3 and 5 were found to be significantly lower than those measured by the colorimetric method, while vaccine lot no. 4 DCA content was essentially the same within experimental error. Standard errors for the colorimetric measurements were repeatedly higher than those for LC. Colorimetric methods are generally recognized as lacking

Table 1
Deoxycholic acid to cholic acid peak area ratios from HPLC analysis of replicate injections of 25 μg ml^{-1} standard assay solution

	25 μg ml^{-1}	
	Intra-day	Inter-day
	0.106	0.113
	0.105	0.111
	0.103	0.109
	0.109	0.117
	0.114	0.121
	0.110	0.112
	0.110	0.109
	0.110	0.109
	0.105	0.112
	0.104	0.109
		0.106
Mean	0.108	0.112
RSD(%)	±3	±4

Table 2
Deoxycholic acid (DCA) content of influenza vaccine samples analysed by LC and colorimetric methods

Vaccine lot no.	LC method	Colorimetric method
3	16.4 ± 0.4	27.8 ± 1.7
4	11.3 ± 0.3	12.3 ± 1.7
5	10.1 ± 0.8	18.8 ± 2.1

Units = μg ml^{-1}.
Values ±SEM.

Table 3
Variation of deoxycholic acid to cholic acid peak area ratio for replicate assays of vaccine samples

	Vaccine lot no. 3 Intra-day	Vaccine lot no. 4 Inter-day (pooled)	Vaccine lot no. 5 Inter-day
	0.0649	0.0483	0.0541
	0.0671	0.0524	0.0520
	0.0749	0.0450	0.0481
	0.0673	0.0519	0.0476
	0.0739	0.0432	0.0337
			0.0378
Mean	0.0704	0.0492	0.0457
RSD(%)	±5.8	±6.6	±16.4

complete specificity [13], whereby factors such as the instability of the coloured complex, the presence of small amounts of impurities or variations in the reaction time and temperature can affect the results obtained. Furthermore, the DCA levels were calculated by extrapolation below the level of the lowest standard used in the standard curve. The linearity of the method below 25 $\mu g\ ml^{-1}$ has not been demonstrated, hence the accuracy of these results could not be established. These factors are likely to contribute to the lack of accuracy and precision of the colorimetric method.

The reproducibility of the LC method for vaccine analysis was examined by assaying vaccine samples for intra- and inter-day variations (Table 3). Five replicate injections of vaccine lot no. 3 on the same day showed acceptable variability, with a relative standard deviation of ±5.8%. For inter-day experiments, same lot ampoules of vaccine lot no. 4 were pooled together and assayed for five consecutive days while individual ampoules of vaccine lot no. 5 were examined. The variability of the assay for the unpooled vaccine (RSD ± 16.4%) was found to be greater than that for the pooled vaccine (RSD ± 6.6%). This data also indicated that method variation was smaller than vial variation. The source of the vial variation is not known at this time.

Conclusion

Deoxycholate, a process-related impurity present in some influenza vaccine preparations, was assayed using a reversed-phase LC method. The LC method described herein is a sensitive and specific method suitable for the detection and quantitation of low levels of deoxycholate in split influenza vaccine. Test parameters commonly used for method validation were found to be acceptable. Larger variations in the DCA content were observed when assaying individual same-lot ampoules when compared to pooled ones.

Acknowledgements — We would like to thank Dr Normand Beaulieu for helpful discussions.

References

[1] C.M. Brand and J.J. Skehel, *Nature New Biol.* **238**, 145–147 (1972).
[2] F.M. Davenport, A.V. Hennessy, F.M. Brandon, R.G. Webster, C.D. Barrett and G.O. Lease, *J. Lab. Clin. Med.* **63**, 5–13 (1964).
[3] A.R. Neurath and B.A. Rubin, in *Monographs in Virology* (J.L. Melnick, Ed.), Vol. 4. Karger, Basel (1971).
[4] R.G. Webster and W.G. Laver, *J. Immunol.* **96**, 596–605 (1956).
[5] A.E. Duxbury, A.W. Hampson and J.G.M. Sievers, *J. Immunol.* **101**, 62–67 (1968).
[6] F.M. Warburton and A.E. Duxbury, International Symposium on Influenza Vaccines for Men and Horses, London 1972; *Symp. Series Immunobiol. Standard* **20**, 92–97 (1973).
[7] F.M. Warburton, *Bull. Wld. Hlth. Org.* **41**, 639–641 (1969).
[8] W.G. Laver and R.G. Webster, *Virology* **69**, 511–522 (1976).
[9] C.R. Szalkowski and W.J. Mader, *J. Anal. Chem.* **24**, 1602–1604 (1952).
[10] J. Goto and T. Nambara, in *Liquid Chromatography in Biomedical Analysis*, Journal of Chromatography Library, Vol. 50 (T. Hanai, Ed.) pp. 81–108 Elsevier (1991).
[11] J. Goto, H. Kato, Y. Saruta and T. Nambara, *J. Liq. Chromatogr.* **3**, 991–1003 (1980).
[12] S. Scalia, P. Pazzi and M. Guarneri, *Anal. Lett.* **22**, 915–927 (1989).
[13] J. Bartos and M. Pesez, *Colorimetric and Fluorimetric Analysis of Steroids*, 274 pp. Academic Press, London (1976).

[Received for review 19 July 1993; revised manuscript received 9 December 1993]