Journal of Chromatography, 631 (1993) 221–226
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2615

Reversed-phase high-performance liquid chromatography of the cardiac glycoside LNF-209 with refractive index detection

Paul H. Zoutendam, David L. Berry and Douglas W. Carkuff

Procter and Gamble Pharmaceuticals, P.O. Box 191, Norwich, NY 13815 (USA)

ABSTRACT

LNF-209 is a glycoside, similar to digoxin, which has potential for use in the treatment of congestive heart failure. However, unlike digoxin it exhibits virtually no useful UV absorption spectra, making detection difficult. One means of detection is the refractive index detector, but like most bulk property detectors it has certain limitations. Its sensitivity is limited and it is sensitive to small changes in a number of parameters, such as temperature, mobile phase composition, and flow-rate. These parameters must be closely controlled to obtain a stable baseline. This paper describes the steps taken to control the system and the development and validation of an assay for LNF-209 in dosing solutions. The method developed is capable of quantitating LNF-209 in solutions of sterile water and 5% dextrose at concentrations ranging from 8 to 6000 μg/ml. The method is linear over this range and quantitative recovery is obtained. The overall average relative standard deviation for replicate analysis of several samples at various concentrations assayed over two days was 2.3%.

INTRODUCTION

LNF-209 is a cardiac glycoside similar to digoxin which has potential for use in the treatment of congestive heart failure [1,2]. A method was needed to determine levels of this aminosteroid in dosing solutions in sterile water and 5% dextrose (D5W). These dosing solutions would be used in animal toxicology studies. Concentrations used would span a very broad concentration range (10–5000 μg/ml). An assay with sufficient sensitivity to accurately quantitate LNF-209 at 10 μg/ml was needed. The most widely used and accepted method of determining digoxin is reversed-phase HPLC with UV detection at 220 nm [3–7]. A simple unbuffered acetonitrile–water mobile phase and a ODS column are most often used [3]. This compound, unlike digoxin, exhibits virtually no useful UV absorption spectra. This makes detection of LNF-209 difficult. A variety of means of detecting LNF-209 have been evaluated. One possibility of doing so, is to use refractive index detection. Refractive index detection, like most bulk property detectors, has certain advantages and disadvantages [8–10]. The advantages of RI detection are that it is a universal method of detection and that it gives similar responses for similar compounds. The disadvantages of RI detection are that it has poor sensitivity and that it is a universal method of detection.

One of the major advantages and disadvantages of RI detection is the fact that it is a universal method of detection. All kinds of solutes can be detected, however, small changes in the temperature, pressure, flow-rate, or mobile phase composition will also be detected, often as noise and/or drift. To accurately quantitate LNF-209 at the low end of the desired concentration range, the refractive index detector is operated on a setting that corresponds to $2.0 \cdot 10^{-7}$ refractive index units (RIU) full scale. For methanol, a change in temperature of 0.005°C or a change in pressure of 0.68 p.s.i. corresponds to a change of $2 \cdot 10^{-6}$ in refractive index [8]. For a methanol–water mixture, a change of 0.04% in the meth-

Correspondence to: P. H. Zoutendam, Procter and Gamble Pharmaceuticals, P.O. Box 191, Norwich, NY 13815, USA.

anol–water ratio corresponds to a change of $2.0 \cdot 10^{-6}$ in refractive index. Hence, to achieve detection at useful levels of sensitivity these variables must be closely controlled, *i.e.* maintained constant. A variety of methods of controlling these variables have been discussed in the literature [8–11]. In order to reduce the noise and drift to acceptable levels for this application, it was necessary to utilize almost all of the recommended procedures. The steps that were taken are summarized below.

Control the temperature of the column. (Use a column heater.)
Control the temperature of the detector cell. (Use a detector with a thermostated cell.)
Continuously stir the mobile phase.
Use a properly operating low pulsation pump. Using an extra pulse dampener is often helpful.
Control the gas content of the mobile phase. Either use helium sparging or use air-saturated mobile phase. (In our hands, helium sparging give slightly superior results.)
Autozero the detector at the start of each injection.

EXPERIMENTAL

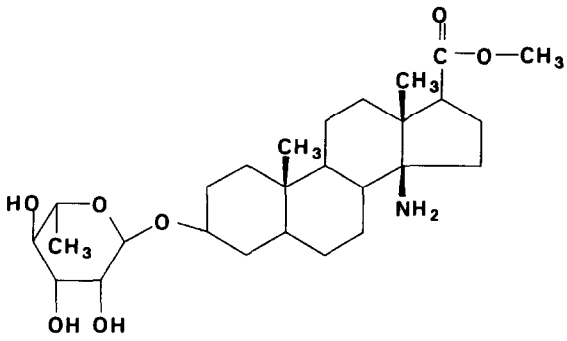
The HPLC system consisted of a Waters 510 pump or a Beckman 100A pump, a Perkin-Elmer ISS-100 autosampler or a Waters WISP 712 autosampler, a Waters Model 410 Differential Refractometer with a column heater, and a Linear Model 1201 recorder. Data was collected and integrated on a Hewlett-Packard LDS data system. An Orion Model 520 pH meter with glass combination electrodes was used to adjust the pH. All reagents and buffers were reagent or HPLC grade. Solvents were HPLC or Spectro grade. Water purified via a Millipore Milli-Q system was used in all cases.

The column was a Spherisorb C_8 column (250 × 4.6 mm, I.D., 5 μm). The mobile phase consisted of 560 ml purified water, 440 ml methanol, 3.4 ml phosphoric acid, and 8.0 g ammonium nitrate. The apparent pH of the mobile phase was adjusted to 3.0 ± 0.1 with ammonium hydroxide. The flow-rate was 1.0 ml/min. The detector and column heater were maintained at 35°C. The detector sensitivity was set to 256 and the scale fractor was 10. An injection volume of 100 μl was used. The salt-enriched mobile phase was prepared by adding five times as much phosphoric acid and ammonium nitrate as was in the mobile phase to a 56:44 mixture of water and methanol. The pH was adjusted to 3.0 ± 0.1 with ammonium hydroxide.

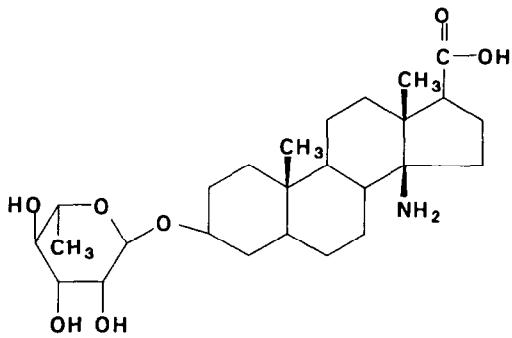
The hydrochloride salt form of LNF-209 is hygroscopic, while the free base form is not. Standards were prepared using the free base form. Samples were quantitated *versus* a standard curve. Standards were prepared in mobile phase at concentrations of 6, 12, 25, 40 and 60 μg/ml. Samples with concentrations above the range of the standard curve were simply diluted with mobile phase until they were within the range of the standard curve. Low-concentration samples (<60 μg/ml) were mixed 4:1 with salt-enriched mobile phase. The use of the salt-enriched solution was found necessary to obtain quantitative recovery.

RESULTS AND DISCUSSION

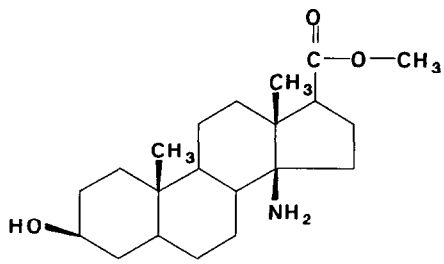
On a typical reversed-phase HPLC system, this aminosteroid gives rather broad, tailing peaks. This is due to the presence of an amine group. See Fig. 1 for the structures of LNF-209 and its potential degradation products. Amine groups are known to cause tailing via interaction with the surface silanols. A large number of mobile phase additives, solvents, columns, etc. were investigated concerning their effect on the chromatographic behavior of these compounds. It was found that the peak shape could be improved by adding a high concentration of cations to the mobile phase. This is illustrated in Fig. 2, which shows the effect of adding ammonium nitrate on the number of theoretical plates, N, and the tailing factor [USP Tailing Factor, 12] of LNF-209. Adding 100 mM ammonium nitrate to the mobile phase is effective at improving the peak shape. Methanol was selected for use as the organic solvent in the mobile phase, because better peak shape was obtained with its use as compared to using acetonitrile. It is of note that the chromatographic behavior of this compound is significantly different when acetonitrile is used in the mobile phase. On a C_8 column with a mobile phase consisting of methanol–water (45:55), LNF-209 eluted with a capacity factor of about 5. If acetonitrile is substituted for methanol in the mobile phase in the same proportion, no peak is observed. The compound is completely retained on the column. If the percentage of acetonitrile is reduced to 25% and triethylamine (7 ml/l) is added to the mobile phase, LNF-209 elutes, but it is barely retained and is not completely resolved from the void volume disturb-



LNF-209



Free acid



aglycone

Fig. 1. Structure of LNF-209 and related compounds.

ance. In a typical reversed-phase application, methanol and acetonitrile normally give nearly equivalent results. This is not the case here. The reason for this unusual behavior is that LNF-209 is highly soluble in methanol, but has very limited solubility in acetonitrile.

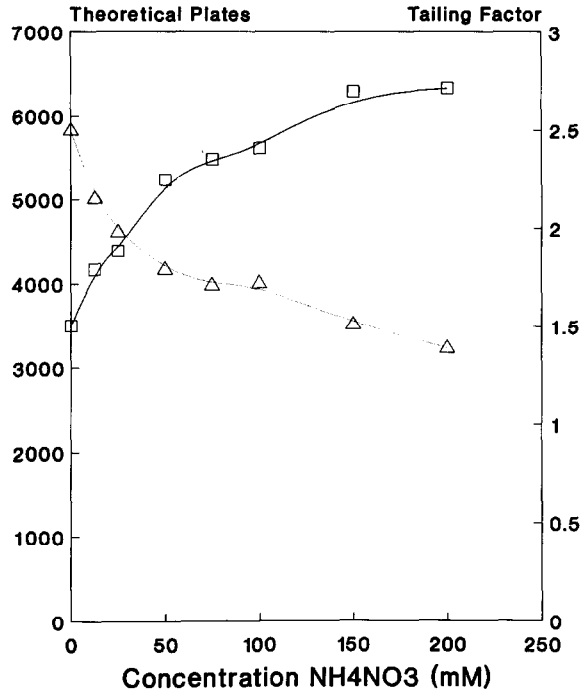


Fig. 2. Effect of adding ammonium nitrate to mobile phase on (□) the number of theoretical plates and (△) the tailing factor,

The HPLC system developed separates LNF-209 from its potential degradation products, the free acid and the aglycone. A chromatogram illustrating this is shown in Fig. 3. The pH of the mobile phase

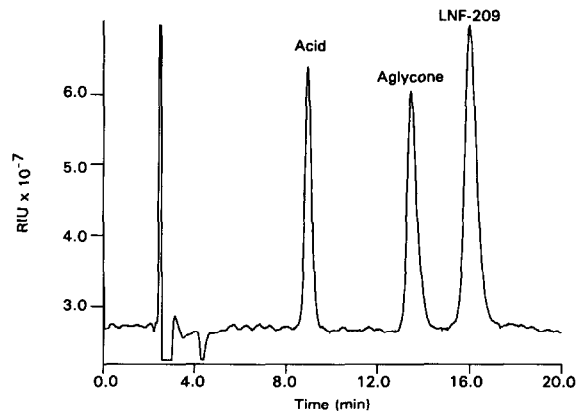


Fig. 3. Chromatogram of LNF-209 spiked with the free acid and the aglycone. Column: Spherisorb C_8 (250 × 4.6 mm I.D., 5 μm); flow-rate: 1.0 ml/min; mobile phase: 560 ml water, 440 ml methanol, 3.4 ml phosphate acid, 8 g ammonium nitrate, pH adjusted to 3.0 with ammonium hydroxide. See Experimental section for additional conditions.

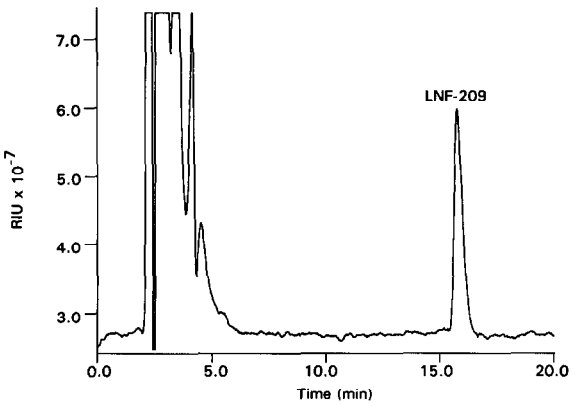


Fig. 4. Chromatogram of a sample preparation of a 36 μg/ml solution of LNF-209 in 5% dextrose. The conditions were identical to those described in Fig. 3.

must be adjusted to 3.0 to insure that the free acid is protonated and retained. At higher pH it elutes in the void volume. A chromatogram of a typical sample preparation for a solution of LNF-209 in 5% dextrose (D5W) is shown in Fig. 4. Dextrose elutes just after the void volume. It produces a large peak, but it is well separated from the drug peak and does not interfere with the analysis.

The linearity of the system was examined by preparing a series of solutions of LNF-209 in mobile phase and injecting them. The solutions were prepared via serial dilution and ranged in concentration from 2–500 μg/ml. Concentration *versus* peak area is linear over the entire range studied (slope = 38961, intercept = −38905, correlation coefficient = 0.9998). Concentration *versus* peak height is not linear over the entire range. At higher concentrations, the line falls off. Over the range from 2–100 μg/ml, concentration *versus* height is linear. The reduction in peak height response at high concentrations is due to increasing peak tailing. Peak tailing increases as more compound is loaded on the column. An injection volume of 100 μl was used for this study, therefore the concentrations injected correspond to 0.2 to 50 μg injected on column. Peak tailing continuously increases as more material is injected on column, however the increase is very gradual from 0.2 to 5.0 μg. Tailing increases more rapidly as the amount injected in column increases from 10 to 50 μg and the plot of concentration *versus* peak height begins to fall off in this range. Even though, the working concentration for this assay was selected to be in the range where the system is linear by both peak areas and peak heights, peak areas were used for all investigations. The limit of detection of the method was also investigated. The limit of detection for LNF-209, where this is defined as a peak corresponding to three times the noise level, was estimated to be 1 μg/ml (or 0.1 μg injected on column).

When solutions of LNF-209 in water or D5W at very low concentrations (8–30 μg/ml) were injected directly into the HPLC, complete recovery was not obtained. Quantitative recovery was obtained for samples containing more than 30 μg/ml. Very high concentration (> 60 μg/ml) samples which were diluted 5:1 or greater with mobile phase also gave quantitative recoveries. Simply diluting the low concentration samples with mobile phase was not an option as this would result in final sample concentrations that were below the level where the RI detector could accurately quantitate this material. Several sample preparation methods designed to provide quantitative recovery at low concentrations were evaluated. It was found that adding 2.0 ml of a salt-enriched mobile phase to 8.0 ml of sample resulted in quantitative recoveries. The salt-enriched mobile phase contained five times the concentration of phosphoric acid and ammonium nitrate that was in the mobile phase and its pH was adjusted to 3.0 with ammonium hydroxide. Hence, when this was added to an aqueous solution, the resultant solution had the same buffer and salt concentration as the mobile phase. This procedure results in a minimal dilution of the sample. This procedure was used for samples in the lowest concentration range. Samples with higher concentrations were simply diluted with mobile phase and injected.

The precision of this method was estimated by preparing a series of solutions in either water or D5W and assaying each solution in triplicate. The concentrations of the solutions spanned the entire concentration range of this assay. The experiment was repeated on a second day by a different analyst. The average result and the relative standard deviation for the three determinations of each solution for samples prepared in water and 5% dextrose are listed in Tables I and II, respectively. The average R.S.D. values in water for days 1 and 2 were 2.53% and 2.10%, respectively. The average R.S.D. values

TABLE I

TRIPLICATE ASSAYS OF LNF-209 IN DOSING SOLUTIONS IN WATER

Based on peak area measurements.

Day 1		Day 2	
Average (μg/ml)	R.S.D. (%)	Average (μg/ml)	R.S.D. (%)
9.0	3.33	8.5	5.02
16.4	2.18	15.9	2.3
35.0	1.98	34.5	0.66
57.3	2.54	59.2	0.22
89.5	2.22	87.1	0.55
292	0.98	312	0.95
359	1.81	357	0.58
588	2.53	586	6.90
740	2.51	752	2.01
1439	3.07	1445	2.70
1929	4.65	1895	3.00
5752	2.52	6000	0.26
Average	2.53	Average	2.10

TABLE II

TRIPLICATE ASSAYS OF LNF-209 DOSING SOLUTIONS IN 5% DEXTROSE

Based on peak area measurements.

Day 1		Day 2	
Average (μg/ml)	R.S.D. (%)	Average (μg/ml)	R.S.D. (%)
7.9	2.37	9.1	5.73
15.6	1.48	16.0	1.26
34.7	3.48	33.8	2.26
60.2	2.29	34.3	3.45
89.8	1.72	91.6	4.26
304	2.53	295	1.72
352	4.95	358	0.54
590	2.13	609	1.79
733	2.63	748	1.18
1547	3.82	1484	1.83
1975	0.71	1998	0.25
6043	2.56	6014	0.53
Average	2.56	Average	2.07

in D5W for days 1 and 2 were 2.56% and 2.07%, respectively.

A spiked recovery study was performed to assess the accuracy and linearity of this method. Solutions containing known concentrations of LNF-209 in water and D5W were prepared. The concentrations of the solutions spanned the entire concentration range of this assay. The samples were assayed and the amount recovered was determined. The experiment was repeated a second time at each concentration by a different analyst. Results for samples in water and D5W are summarized in Tables III and IV, respectively. The overall average recoveries were 100.1% and 99.1% for samples in water and D5W, respectively. Linear regressions were performed on the amount added *versus* the amount

TABLE III

LINEARITY AND RECOVERY OF LNF-209 IN WATER

Based on peak area measurements.

	Day 1	Day 2	Day 3
Range (μg/ml)	8–6076	8–300	298–5830
n	16	9	9
Average recovery (%)	100.5	101.3	98.0
R.S.D. (%)	2.39	1.82	1.22
Results of linear regression for plots of amount added vs. amount found			
Slope	1.0045	1.0261	0.9789
Standard error slope	0.0036	0.0053	0.0024
Intercept	5.0616	−0.5369	−2.1255
Standard error intercept	6.3699	0.7699	0.0024
Correlation coefficient	0.99991	0.99990	0.99998

TABLE IV

LINEARITY AND RECOVERY OF LNF-209 IN 5% DEXTROSE

Based on peak area measurements.

	Day 1	Day 2	Day 3
Range (μg/ml)	8–6180	8–294	280–5950
n	24	10	9
Average recovery (%)	98.9	99.6	99.2
R.S.D. (%)	3.17	1.26	2.24
Results of linear regression for plots of amount added vs. amount found			
Slope	1.0408	0.9966	1.0006
Standard error slope	0.0031	0.0019	0.0090
Intercept	−10.7775	−0.0213	−4.7151
Standard error intercept	4.6411	0.2798	21.326
Correlation coefficient	0.99990	0.99985	0.99971

found. The results of the regression analysis are summarized in Tables III and IV. The results indicate that this assay in linear in nature.

CONCLUSIONS

Refractive index detection has been found to be a reliable means of quantitating LNF-209, an aminosteroid with little to no UV absorption. By controlling the temperature of the system and the composition of the mobile phase, adequate sensitivity for detecting LNF-209 at the levels required for this application was achieved. The assay developed is capable of accurately quantitating this material in dosing solutions in water and 5% dextrose over a broad concentration range. The HPLC system developed gives well shaped peaks and is capable of separating the major component from its potential degradation products, the acid and the aglycone. The addition of a high concentration of cations to the mobile phase was found to be effective at improving the peak shape and reducing peak tailing.

REFERENCES

1 L. Brown, *Clin. Exp. Pharmacol. Physiol.*, 17 (1990) 589.
2 D. Rocher and P. Lechat, *J. Mol. Cell Cardiol*, 23 (1991) Supplement IV, S52, no. 156.
3 *USP XXII–NSF XVII*, US Pharmacopeial Convention, Rockville, MD, 1990, p. 438.
4 F. Erni and R. W. Frei, *J. Chromatogr.*, 130 (1977) 169.
5 Y. Fujii, Y. Ikeda and M. Yamazaki, *J. Chromatogr. Sci.*, 28 (1980) 288.
6 B. Desta and K. M. McErlane, *J. Pharm. Sci.*, 71 (1982) 1018.
7 Y. Fujii, Y. Ikeda and M. Yamazaki, *J. Chromatogr.*, 448 (1988) 157.
8 M. N. Munk, in T. M. Vickrey (Editor), *Liquid Chromatography Detectors*, Marcel Dekker, New York, 1983, Ch. 6, p. 165.
9 R. P. W. Scott, *Liquid Chromatography Detectors*, Elsevier, Amsterdam, 2nd ed., 1986, Ch. 3, p. 49.
10 E. S. Yeung, *Detectors for Liquid Chromatography*, Wiley, New York, 1986, Ch. 1, p. 1.
11 W. A. Dark, *J. Chromatogr. Sci.*, 24 (1986) 495.
12 *USP XXII–NF XVII*, US Pharmacopeial Convention, Rockville, MD, 1990, p. 1567.