Development of a High-Performance Liquid Chromatographic Assay for G418 Sulfate (Geneticin)

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We have developed a chromatographic assay with high sensitivity and specificity to quantify G418 sulfate (Geneticin), an antibiotic used routinely in molecular genetics experiments for selecting eukaryotic transformants. With this method, G418 in tissues and plasma samples can be quantitated without the confounding factors often associated with biological assays. After removal of proteins in homogenized tissue or plasma samples with methanol (2:1, vol/vol), the amino group of G418 was derivatized with 1-fluoro-2,4-dinitrobenzene (DNFB) to form the UV-visible G418-DNFB product. The DNFB-derivatized G418 was separated on a reversed-phase C_{18} column with an acetonitrile and water gradient as the mobile phase. Under these assay conditions, the detection limit for G418 sulfate in buffer, plasma, and tissues was recorded at 78 ng/ml and the linearity was recorded for concentrations up to 100 µg/ml. The data obtained from this analysis indicate that this assay can be used for the quantitative determination of G418 sulfate in plasma and tissue samples.

G418 sulfate (Geneticin), a 2-deoxystreptamine antibiotic produced by *Microspora rhodorangea*, is structurally related to the aminoglycoside gentamicin but has inhibitory activity against a greater variety of pro- and eukaryotic organisms (9, 12). This antibiotic has been tested as an antiparasitic agent; specifically, it has shown potent antiamoebic activity. Its clinical use as an antiparasitic agent has been limited, however, by its significant toxicity in host animals and parasitic cells. With the increased use of bacterial plasmids which contain neomycin resistance determinants to introduce foreign genes in human gene therapy, the use of G418 as a drug marker for selecting DNA-transfected cells has become a key factor in the successful development of human gene therapies. Expression of aminoglycoside phosphotransferase 3′ (I or II), the neomycin resistance gene product, in eukaryotic cells enables transfected cells to grow in media containing G418 sulfate (9). The ability of G418 to eliminate nontransformed eukaryotic cells, in contrast to neomycin, which mainly affects prokaryotic cells, has made this antibiotic indispensable for selection of eukaryotic cell transformants (8).

With the advent of human trials in gene therapy, new approaches designed to improve human gene transfer or expression in vivo have become increasingly important. Thus, the need for a selectable marker that can be used in vivo as well as in vitro is evident. Combination use of G418 with neomycin-resistant plasmid determinants is currently one of the most widely used strategies to isolate transfected eukaryotic cells in vitro (11). Also, the recent success in stable expression of foreign gene products of therapeutic interest has been due in part to the transfected cell population containing the resistance genes being increased or maintained by applying selection pressure through the administration of drugs such as G418 sulfate (7, 10). Recent reports on four adenosine deaminase (ADA)-deficient children treated by gene therapy indicated that the gene encoding ADA can be successfully transduced in peripheral blood lymphocytes (4, 5). However, a major limita-

tion with ADA and other gene therapies is the relatively low number of genetically modified cells which are available to alleviate genetic defects in animals or humans (10). Strategies for selecting genetically modified cells are being developed for rapidly dividing cell populations with the selectable human multidrug resistance gene MDR1 (1, 13, 15). For nondividing cells, other means of selection are needed. Toward these goals, we have considered using antibiotic selection with G418 sulfate as a model.

In order to determine if therapeutic and safe concentrations of G418 sulfate are achieved in vivo, it is important to monitor tissue and blood levels of the drug. Although microbiological assays permit an estimation of G418 potency, currently there is no quantitative chemical assay available for characterizing the chemical purity and concentration of G418 within a biological matrix. One of the major limitations in the development of a chemical assay for G418 is its property of poor UV absorption. While G418 can be separated from biological contaminants by high-performance liquid chromatography (HPLC), the poor UV absorption limits its utility. Because G418 contains several primary amines that can be derivatized with a chromophore, we have used Sanger's reagent, 1-fluoro-2,4-dinitrofluorobenzene (DNFB), to dinitrophenylate these amines in the development of a quantitative HPLC assay for G418 (14). With this reversed-phase HPLC assay, we found that DNFB-derivatized G418 can be sensitively and selectively detected in biological samples.

MATERIALS AND METHODS

Reagents. G418 sulfate was purchased from GIBCO BRL, Gaithersburg, Md.; Sanger's reagent, DNFB, was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wis.; and HPLC-grade methanol and acetonitrile were purchased from J. T. Baker, Phillipsburg, N.J. All other reagents were of analytical or higher grade.

Sample preparation and derivatization. (i) G418 sulfate in buffer. For construction of initial standard curves, a stock solution of G418 sulfate with a concentration of 10 mg/ml was prepared daily in 0.02 M borate buffer, pH 8 (henceforth referred to as borate buffer). This stock solution was diluted with borate buffer to achieve a final concentration range of 0 to 1.5 µg/ml. Dilutions were done in quadruplicate for each run to determine run-to-run and day-to-day variation.

(ii) G418 sulfate in tissues. This standard curve for G418 sulfate was prepared by the addition of 100 µl of G418 sulfate in borate buffer to 100 µl of unclarified homogenized mouse tissue or serum. To detect G418 in the livers of mice to

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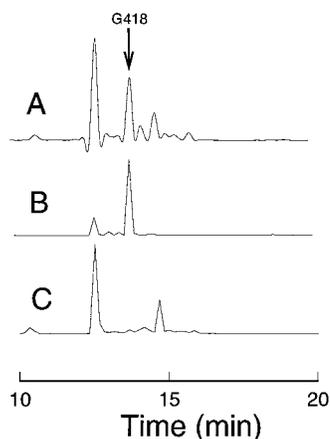
FIG. 1. Representative chromatograms for G418 derivatized with DNFB. G418 in either liver homogenate (A) or borate buffer (B) or a liver homogenate control (without G418) (C) was deproteinated, derivatized with DNFB, and chromatographed as described in Materials and Methods. The concentration of G418 sulfate was 2.5 μg/ml for panels A and B.

which this compound had been administered, the tissue samples were first homogenized in the presence of borate buffer (1 g of tissue per 5 ml of buffer) with a motor-driven Potter-Elvehjem-style tissue homogenizer (VWR, Seattle, Wash.) operating at 300 rpm for 1 min.

To detect G418 in tissue and plasma specimens, 100 μl of each G418-containing sample was deproteinized with either absolute methanol (1:2 [vol/vol], tissue homogenate:methanol) or 6% (wt/vol) trichloroacetic acid (TCA). Protein precipitate was separated from the supernatant by centrifugation in a model 15 Biofuge (Baxter Scientific Products, McGraw Park, Ill.) at $15,000 \times g$ for 10 min. The G418 in the supernatant was lyophilized and reconstituted in 50 μl of borate buffer. It was then used for precolumn derivatization as described below.

(iii) Derivatization of G418 with DNFB. The samples containing G418, either from the standards in buffer or from deproteinated tissue homogenates, in 50 μl of borate buffer were each mixed with 150 μl of 0.15 M DNFB and incubated at 100°C for 45 min. At the end of the incubation, the liquid from the samples was completely evaporated. The samples, cooled to room temperature, were each dissolved in 500 μl of acetonitrile-water (1:1, vol/vol). Then 200-μl aliquots of these samples were transferred to 300-μl glass vial inserts (Alltech Associates [part number 98118], Deerfield, Ill.), and 50-μl aliquots were injected into an HPLC system fitted with an autoinjector (Waters Corporation, Milford, Mass.).
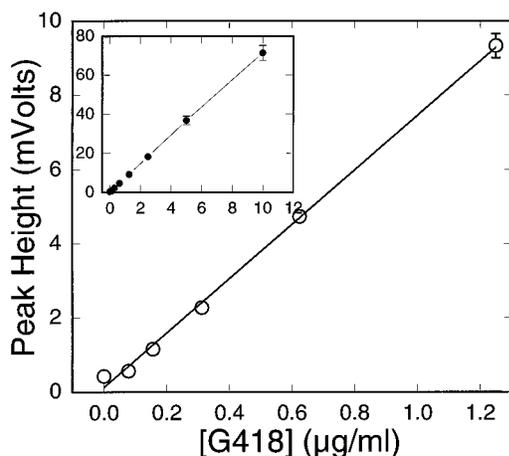

FIG. 2. Linearity and detection limit for the HPLC assay detecting G418 derivatized with DNFB. The indicated concentrations of G418 sulfate in borate buffer were chromatographed as described in Materials and Methods and analyzed based on the peak height detected for the DNFB-derivatized G418. Each data point represents the mean ± the standard deviation of results from quadruplicate samples. The figure insert shows a wider linear concentration range (0 to 12 μg/ml) of G418 standards.

TABLE 1. Precision of the HPLC assay for G418 sulfate[a]

Type of variation	Retention time (min)	Slope (mV/μg)
Run to run	13.76 ± 0.02[b]	438.8 ± 1.9[b]
Day to day	13.70 ± 0.09[c]	424.9 ± 14.9[c]

[a] The intraday and interday variation for the assay were determined from analysis of standard curves of G418 sulfate (0 to 3 μg/ml) spiked into borate buffer. The resulting retention times and slopes of peak heights were used to evaluate the variation of the assay.
[b] Values represent the averages ± the standard deviations of data from four runs of standard curves containing G418 sulfate (0 to 3 μg/ml) spiked into borate buffer.
[c] Values represent the means ± the standard deviations of data from five runs of standard curves prepared as described above but analyzed on separate days.

DNFB-derivatized G418 samples were stored in the dark, where they remained stable at room temperature for 1 week.

Chromatographic conditions and instrumentation. The DNFB-derivatized G418 sulfate was separated on a reversed-phase HPLC system (Waters Corporation). The samples were introduced with a WISP model 712 autosampler. Separation of the G418-DNFB conjugate was performed with a reversed-phase C_{18} column (8 by 100 mm with a 5-mm internal diameter; catalog number 85721, Waters Corporation) at room temperature. Elution was achieved with a linear gradient, with acetonitrile-water as the mobile phase.

Mobile phases consisted of solvent A, acetonitrile and water (50:50), and solvent B, acetonitrile. Both solvents were prefiltered with a 0.45-μm-pore-size nylon membrane (Alltech Associates) to reduce interference. With the Maxima 820 computer-controlled workstation (version 3.3; Millipore Corp., Milford, Mass.), the solvent flow rate was set at 1 ml/min. The linear gradient between the two mobile phases was set as follows: (i) from 0 to 2 min, 100% solvent A; (ii) from 2 to 6 min, a linear increase from 0 to 100% solvent B; (iii) from 8 to 12 min, 100% solvent B; (iv) from 12 to 12.5 min, return to 100% solvent A; and, finally, (v) from 12.5 to 25 min, reequilibration of the column with 100% solvent A. Using a UV detector (model 440; Waters Corporation) with the wavelength set at 340 nm, DNFB-derivatized G418 sulfate was eluted with a retention time of 13.60 ± 0.09 min.

Data analysis. The chromatograms were recorded and analyzed by the Maxima 820 computer workstation. For initial experiments, the linearity of the assay was determined by both peak height and peak area response. We found that both methods produced similar results; hence, only peak height data are presented in this report. Slopes of the standard curves were fitted by least-squares linear regression. Statistical comparisons of the standard curves were performed with a two-sided Student t test with a significance level of 0.05.

RESULTS AND DISCUSSION

We first determined whether derivatization of G418 sulfate with DNFB would provide a chromophore sufficient for UV detection of G418 by a reversed-phase HPLC system. Based on the method developed for amikacin by Barends et al. (2), DNFB-derivitized G418 was eluted isocratically with an acetonitrile-water mobile phase. We found, however, that an isocratic mobile phase was not sufficient to separate G418 from other coeluting DNFB-derivatized components found in mouse tissue and plasma samples.

Therefore, we developed a linear gradient system, increasing the acetonitrile concentration from 50 to 100% to adequately separate the DNFB-derivatized G418 from contaminants. Under these conditions, G418 eluted with a retention time of 13.6 ± 0.09 min. Typical chromatograms are presented in Fig.

TABLE 2. Recovery of G418 in two protein precipitation procedures[a]

Method	Recovery	% CV
TCA	65.6 ± 8.2[b]	12.5
Methanol	81.8 ± 5.1[b]	6.3

[a] The values here represent percent recovery for four runs of TCA and methanol methods in comparison with standard curves for 0.5, 1.0, and 2.0 μg of nonprecipitated material.
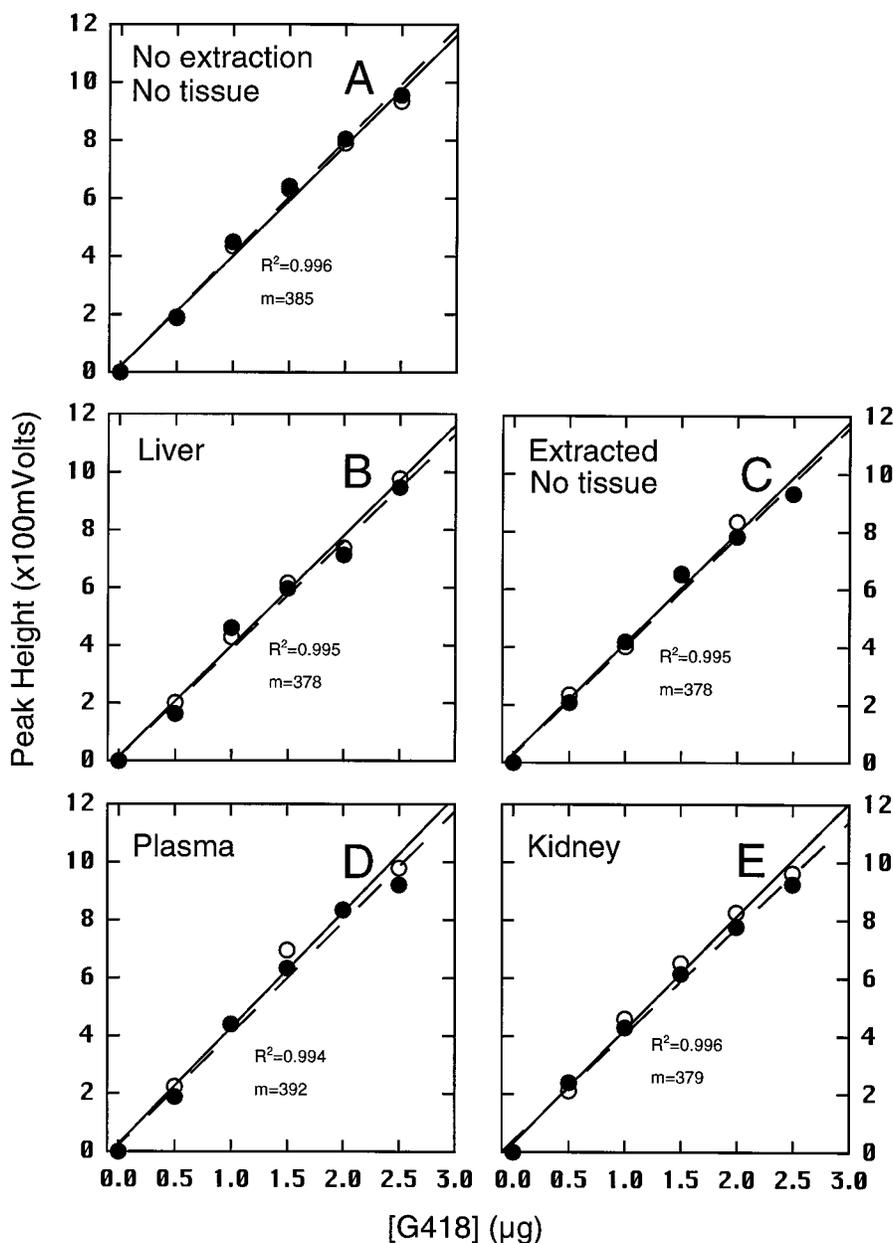[b] $P < 0.016$ (t test) for the two methods.

FIG. 3. Detection of G418 in mouse tissues. The various concentrations of G418 sulfate in liver homogenate (B), borate buffer (C), plasma (D), or kidney homogenate (E) were deproteinated with methanol, derivatized, and assayed for G418 by the HPLC assay as described in the text. In parallel, as a control to detect the effect of the deproteination procedure, G418 sulfate in borate buffer was derivatized without methanol deproteination (A). Open and closed circles represent the mean data from run 1 and run 2, respectively.

1. The free, hydrolyzed DNFB eluted near the injection peak; therefore, it did not interfere with G418 sulfate detection. Also, since the concentration of DNFB is always in excess, the derivatization reaction proceeded to completion.

With the G418 sulfate standards prepared in borate buffer, the linearity and detection limit of the assay were determined. Figure 2 shows a mean standard curve constructed from four replicates of varying concentrations of G418 sulfate in borate buffer. Within the concentration range of 0.078 to 10 μg/ml, this HPLC assay exhibited low-level variability and excellent linearity, with a correlation coefficient ($r^2 = 0.999$) based on the resultant peak height analysis of the chromatograms. Linearity was observed at concentrations up to 100 μg/ml (data

not shown). The detection limit, which was greater than 2 standard deviations above the baseline, was estimated to be 78 ng/ml. While peak height signals below 78 ng/ml could be detected, they were not reproducible, and such low concentrations were considered undetectable.

To examine the reproducibility of the assay, we determined intraday and interday variation of samples containing 0.25, 1.5, or 3.0 μg of G418 per ml in borate buffer. These concentrations were chosen based on estimations of levels that would be achieved with our animal studies. Intraday variation was tested on 1 day in quadruplicate, while interday reproducibility was examined over five separate days. As shown in Table 1, both interday and intraday variation were low, with the resulting

coefficient of variation (CV) being below 6% for both. These results indicate that the reproducibility of this assay was excellent.

For the detection of G418 in tissue homogenate or plasma samples, a deproteinization technique is essential to isolate this highly water-soluble compound. Of the various deproteinizing agents used (including methanol, acidified methanol, and acetonitrile) to remove proteins, methanol (1:2 [vol/vol], sample: methanol) or 6% TCA was found to be the most useful. The efficiencies of G418 detection by the methanol and TCA deproteination methods for mouse liver homogenate containing (0- to 20-μg/ml) G418 sulfate are presented in Table 2. The percentage of G418 recovered after protein precipitation was determined from the ratio of the slope from standard curves of G418 prepared in the tissue homogenate compared with that of G418 prepared in borate buffer (without tissue). As can be seen in Table 2, removal of protein with methanol produced a higher (81.8 versus 65.6; $P < 0.016$) percent recovery of G418 with less variation (6.3 versus 12.5% CV) when compared with the TCA method. Therefore, the methanol deproteination method was used subsequently.

By the optimized deproteinization technique, we next determined whether G418 in various tissues can be separated from the biological milieu under the chromatographic conditions described above. A typical chromatogram of G418 recovered from a liver sample is shown in Fig. 1A. Under these assay conditions, the liver homogenate produced minimal background in the region where the DNFB-derivatized G418 peak was detected (Fig. 1C).

Therefore, we used this deproteinization method to determine whether this assay could be used to detect G418 in kidney and plasma samples. As shown in Fig. 3A, the deproteination procedure produces practically the same standard curve as that of neat samples in buffer. Furthermore, the same method can be used to detect G418 in plasma and kidney samples with similar efficiency, which is evident from the similarity of the concentration-dependent peak height responses (slopes, 378 to 392 mV/μg) detected regardless of the tissue being tested. For clinical analysis of G418 in tissues and plasma, an HPLC assay which uses an internal standard, such as neomycin B or C, can be easily incorporated into the current protocol.

Of the various methods used for detecting antibiotics in biological samples, liquid chromatography has been increasingly utilized to determine aminoglycoside levels. In the development of HPLC methods for antibiotics, comparisons and correlations of bulk products are usually made with estimates of antimicrobial agent potencies determined by biological assays. While these biological assays may be more sensitive than HPLC assays, chemical identities cannot be directly discerned without elaborate and time-consuming analysis. With this HPLC assay, we can sensitively and rapidly detect G418 in a quantitative manner. Also, Barends et al. (2) have shown with amikacin that concentrations estimated by HPLC and bioassay are in excellent agreement ($r^2 = 0.993$). In addition, the linearity, sensitivity, and recovery of G418 in this assay match or exceed those for HPLC methods developed for amikacin (2) and gentamicin (3).

In general, HPLC assays for other aminoglycosides utilize pre- or postcolumn derivatization with o-phthalaldehyde to form a fluorogenic compound. Theoretically, G418 can be derivatized with fluorescent reagents such as o-phthalaldehyde by a postcolumn derivatization technique to reduce the detection limit. However, postcolumn derivatization with o-phthalaldehyde may increase baseline noise as well as the cost of detection because the flow cell may be contaminated by reaction products, consumption of the reagent is high, and reaction time can be prolonged only by increased dead volume, resulting in additional peak broadening (6).

In theory, it is possible to use G418 as a means to selectively expand genetically modified cells in vivo in order to increase transgene expression. We have developed and characterized a G418 sulfate HPLC assay with specificity and high sensitivity that is suitable for detection of the drug in biological samples. Our results show good linearity and recovery from tissues in comparison with controls for the concentration range used.

ACKNOWLEDGMENTS

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