Rapid, Inexpensive Quality Control of Fluorine-18 2-Deoxy-2-Fluoro-D-Glucose Preparations Using the Hexokinase Reaction In Vitro

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A rapid enzymatic method for determining the purity of 2FDG preparations has been devised. A small aliquot of the preparation is incubated with a hexokinase/adenosine triphosphate/ Mg^{+2} mixture and passed through a Dowex 1 ion-exchange column, which retains the 2FDG-6-phosphate. Another aliquot, without prior incubation, is passed through an identical column and the 2FDG radioactivity is found in the eluant. The criteria for purity are quantitative retention of the 2FDG-6-phosphate on the column and no retention of 2FDG. Comparison of the HK method with thin layer and high performance liquid chromatography assays indicate that the HK method can serve as a rapid, simple and inexpensive alternative to these other methods. It can be used in a routine quality control program and may be easily adaptable to automated 2FDG synthetic methods.

J Nucl Med 29:1443–1447, 1988

Fluorine-18 2-deoxy-2-fluoro-D-glucose (2FDG) is probably the single most useful PET radiopharmaceutical (*1*). Its utility arises from its ability to quantitate cerebral glucose metabolic rate (CMR_{glu}) using modifications of the 2-deoxyglucose model, which is now well established (*2*). In addition, it has vast potential for cardiac and tumor-scanning studies (*3–6*). Since the first preparation of 2FDG, several synthetic routes, using both electrophilic (*7–9*) and nucleophilic (*10–12*) ^{18}F intermediates, have been published.

Part of the value of cerebral 2FDG studies is that rates of CMR_{glu} are obtained (*13*), and the quantitative nature of these measurements is important. It is thus critical that the purities of 2FDG preparations be high and well defined. The standard approach to quality control is high performance liquid chromatography (HPLC). There are several potential problems with HPLC, however, including coelution of impurities along with 2FDG, retention of impurities on columns, and variability of retention times. These problems can be exacerbated by improper preparation of samples, improper care of columns or preparation of mobile phases, and failure to recognize deterioration of columns or malfunction of other components of the HPLC system. These issues are the province of radiochemists, and properly staffed laboratories, including the current "research" positron emission tomography (PET) centers, are able to use HPLC effectively.

Newly established "clinical" or "turn-key" PET centers, however, operating with technologists and automated synthetic equipment, may wish to avoid the expense and potential shortcomings of HPLC. Enzymatic methods are commonly employed alternatives to chromatographic analysis of metabolically active compounds (*14*). The essence of the 2FDG method is measurement in vivo of the flux through the enzyme hexokinase (HK), which transfers a phosphate group from adenosine triphosphate to carbon atom 6 of glucose and a quite restricted range of similar sugars (*15–17*). In vitro assay with HK is also possible, and the 6-phosphates are easily separated from the free sugars with ion-exchange resin (*18,19*).

We therefore decided to evaluate an in vitro assay with hexokinase as the basis of rapid quality control of batches of 2FDG made by reaction of [^{18}F]fluoride with 1,3,4,6-tetraacetyl-2-O-trifluoromethanesulfonyl-β-D-manno-pyranoside (*11*).

Received Oct. 13, 1987; revision accepted Mar. 9, 1988.

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METHODS

Development of Assay

Samples containing chromatographically pure 2FDG (10–100 mCi) were made up to 0.1 ml with water and added to 0.1 ml of Grade 1 100 m*M* disodium adenosine triphosphate (ATP) (Sigma Chemical Co., St. Louis, MO) plus magnesium chloride (adjusted to pH 7.0 with NaOH). After incubation at room temperature for various times with 1 IU of Type C300 HK (Sigma) the mixture was applied to a column (0.7 × 4cm glass pipette) of Dowex 1 (Fisher, Fairlawn, NJ) ion-exchange resin (400 mesh, × 8, chloride-form). The column was washed with water (routinely 4 ml) and ^{18}F activity, in both column and water, was measured in a dose calibrator (Capintec, Ramsey, NJ). Purities were calculated as the amount of ^{18}F activity in the column divided by the total activity. A duplicate sample of each 2FDG preparation was applied to a Dowex 1 column without prior incubation with hexokinase, and purities expressed as the activity in the washings divided by the total activity.

Validation of Assay

Samples of 2FDG preparations were analyzed with HK as described above, by thin-layer chromatography (TLC) (plastic silica gel plates (Macherey-Nagel, FRG) with ethylacetate/ethanol, 4:1, v/v), and by HPLC (HPX-87P column (BioRad, Richmond, CA) eluted with 0.5 ml/min water at 85°). On TLC, spots were located by autoradiography with x-ray film. Plates were then cut into four approximately equal parts comprising: the origin; the 2FDG spot ($R_F \cong 0.5$); from the top of the 2FDG spot to the solvent front; and an area between the 2FDG spot and the origin. The pieces of the TLC plate were assayed, and the percent purity calculated by dividing the activity of the 2FDG spot by the sum of activities of all four fractions (× 100%).

Samples were also spotted for TLC after incubation of 2FDG with HK and ATP. Purities were expressed as the ratio of activity on the origin to the total activity.

Radioactivity eluting from the HPLC column was detected by flowing the mobile phase through a small loop of plastic tubing in a NaI(Tl) well-counter and displaying the amplified signal on a chart recorder. The areas of all peaks were measured and the fraction of the total area in the 2FDG peak calculated.

Preparation of 2FDG

^{18}F was produced from a Cyclotron Corporation CS-15 cyclotron with an oxygen-18 water target as described previously (*20*). For the purposes of this analytical work, several changes were made to the published synthetic method (*11*) for 2FDG. After the reaction of the ^{18}F fluoride with the starting material (Aldrich, Milwaukee, WI) and evaporation of acetonitrile, the mixture was partitioned between water (2 ml) and diethylether (15 ml). The latter was blown to dryness and the residue treated with 1N HCl. The hydrolysis mixture was neutralized by passage down a column of ion-retardation resin and residual ^{18}F fluoride removed with alumina. We refer to this material as the crude product. The solution was finally passed through a SEP-PAK (Waters Chromatography Div., Milford, MA) containing octadecylsilane-bonded silica gel. Samples were taken for analysis from the water and ether fractions after incorporation, and before and after the SEP-PAK treatment. In this way, samples of varying purities were available for testing the enzymatic method of analysis. Furthermore, the fractions taken before the final SEP-PAK treatment are likely to be enriched in impurities which might occasionally contaminate preparations of 2FDG made by the tetraacetylmannose triflate method.

RESULTS AND DISCUSSION

Preliminary tests confirmed that 2FDG (chromatographically pure on HPLC) did not bind to the Dowex 1 columns. Four 1 ml washes with water removed 95% of the ^{18}F. However, 2FDG-6-phosphate did not measurably elute from the columns with water. Early work also showed that the phosphorylation reaction was 95% complete in 2 min under the standard conditions.

Two potential impurities in the 2FDG preparations were also found to bind to Dowex 1 resin. Fluorine-18 fluoride, by virtue of ion-exchange, was completely retained, and tetraacetyl 2FDG, presumably because of hydrophobic interactions, was retained to varying degrees (17–90% in several experiments).

Figure 1 shows chromatograms from representative experiments. TLC plate 1 shows the water layer run in a solvent mixture of low polarity. The spot with the highest R_F value is presumably tetraacetyl 2FDG, since it was the major component in the ether layer (not shown). Unreacted fluoride and 2FDG itself are too polar to migrate in this mobile phase, and thus remain at the origin. The intermediate spots probably represent products of premature partial removal of the acetate protective groups. For plate 2, a more polar solvent mixture was used, where 2FDG migrates but fluoride still remains at the origin. In the left lane, the hydrolyzed reaction product is seen to contain 2FDG ($R_F \cong 0.5$) and also some fluoride (origin) and labeled compounds with higher R_F values. Presumably the latter still contain one or several acetate groups. The center lane, for comparison, was spotted with the original water layer. After final SEP-PAK treatment (lane 3) the 2FDG preparation showed a single spot. Plate 3 was spotted with the same fractions as Plate 2, except that they were first incubated with HK plus ATP/Mg. Note that the 2FDG spot in lane 3 is totally removed, and replaced with label (2FDG-6-phosphate) at the origin.

HPLC of the final product revealed two very small peaks in addition to 2FDG; in the water layer these peaks were two of the several ^{18}F species which passed through the HPLC column (Fig. 2).

The final product passed through Dowex 1 columns with very little retention, but after treatment with ATP and HK retention was almost complete. There was no consistent difference between untreated and HK-treated water layers in terms of behavior on Dowex 1, confirming that the total premature removal of the protective groups does not occur in the reaction mixture to a

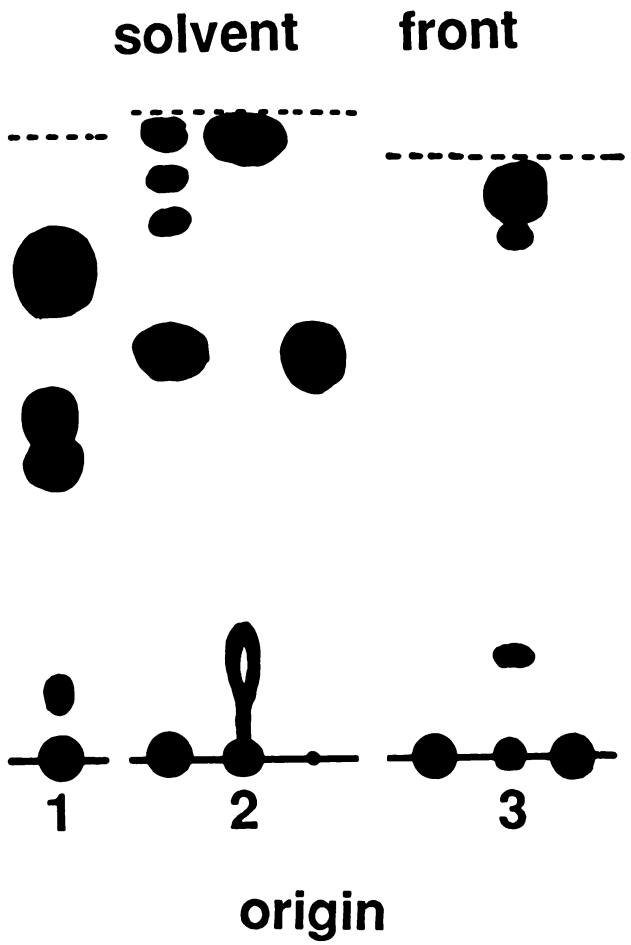

FIGURE 1
Thin layer chromatography of 2 FDG preparations. 1: Run in ethylacetate/hexane (1:2, v/v), reaction mixture; 2: Run in ethyl-acetate/ethanol (4:1, v/v), left to right: crude 2FDG after acid hydrolysis; water layers from incorporation step; 2FDG preparation after passage through C18 SEP-PAK, (3) same as (2), except treated with HK.

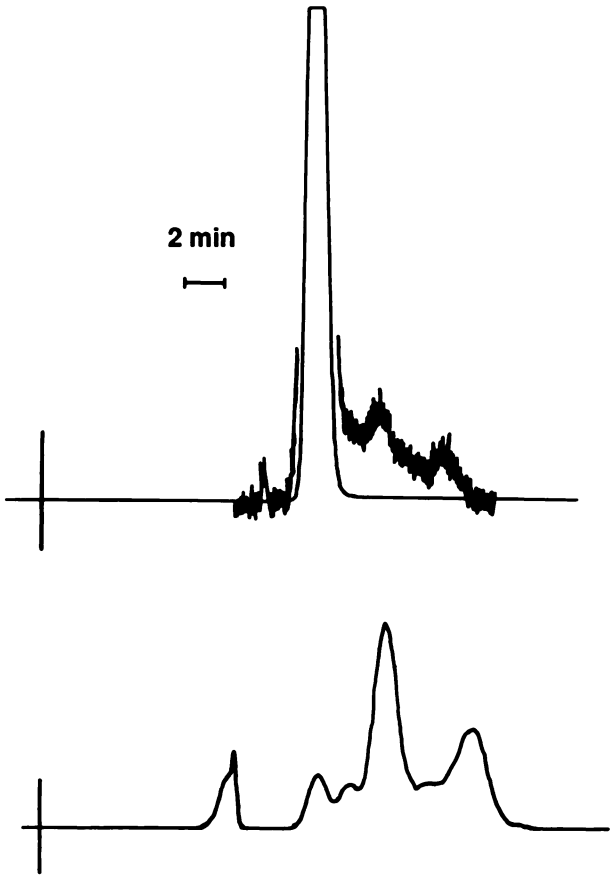

FIGURE 2
HPLC of 2FDG preparations. Top: 2FDG preparation with X100 trace superimposed. Bottom: Water layer from incorporation step.

significant extent. Analytical data for eleven consecutive preparations of 2FDG are shown in Table 1. Mean apparent purities of the final product, before the SEP-PAK treatment, were over 90% for all three methods. HPLC showed the highest apparent purities. After the SEP-PAK treatment the purities given by all methods increased by 1–2%, but this increase was not statistically significant, except for HPLC ($p < 0.05$). A total of seven data points, no more than one from each analytical group and no more than two for a given batch of 2FDG, were excluded because they were more than 3 s.d.s from the mean of the other values. On three of the 11 days TLC was not done, and on two of these days HK was not used.

The purity as determined by TLC was lower because of "tailing" of the 2FDG spot, and also because all of the ^{18}F activity is accounted for in this technique (apart from volatile species). Our HPLC purities are based only on ^{18}F which comes through the column. HPLC purities can also be based on counting a duplicate aliquot of the injected sample, but in practice it is hard to recover 100% of the label because small fractions remain in the needle and injector. We certainly recover >90% of the injected 2FDG. In any analytical system it is difficult to unambiguously assign purities in excess of 95% without using an appropriate internal standard to give precise values for recoveries. The values for apparent purity of SEP-PAK treated 2FDG given in Table 1 are not corrected for the label which remained

TABLE 1
Purities of 2FDG Assessed by Several Different Methods*

Method	– SEP-PAK	+ SEP-PAK
HPLC	97.0 ± 1.5 (11)	99.4 ± 0.6 (10)
TLC	90.2 ± 2.3 (8)	92.3 ± 4.9 (8)
TLC + HK	95.7 ± 2.0 (8)	96.3 ± 3.2 (8)
DOWEX 1	94.3 ± 2.9 (10)	96.1 ± 1.2 (10)
DOWEX 1 + HK	94.6 ± 2.4 (8)	95.5 ± 2.2 (8)

* Values of the mean ± s.d. with the number of replicates in parenthesis.

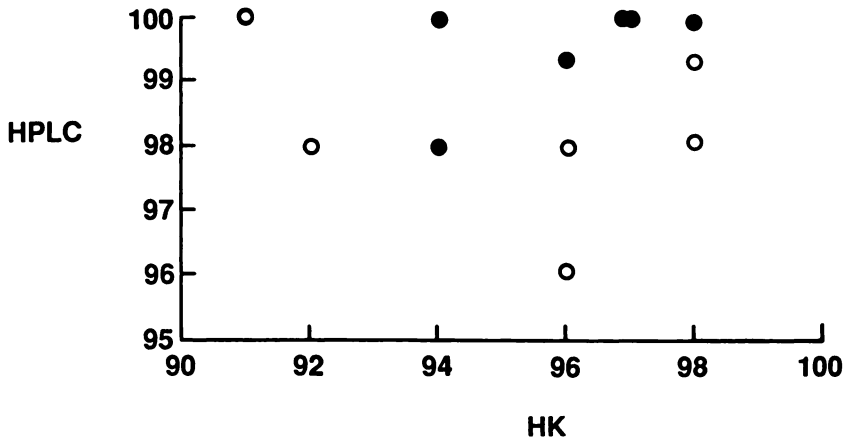

FIGURE 3
Purities of 2FDG preparations as determined by HPLC and the HK method. (○): Passed through C18 SEP-PAK; (●): Crude FDG.

on Dowex 1 columns in the samples which were not incubated with HK. Strictly speaking, this small fraction of the label should be subtracted from the label which binds to the columns after HK treatment because it might represent impurities such as ^{18}F fluoride. Applying this correction to the original data demonstrated unequivocally that the preparations contained at least 92.0 ± 2.5% rapidly phosphorylatable ^{18}F. This is certainly an underestimate, however, because of incomplete wash-out of 2FDG, and incomplete conversion to the 6-phosphate and/or its subsequent trapping. In Figure 3, apparent purities obtained for six batches of 2FDG by the HK method are plotted against those obtained by HPLC. This graph demonstrates that slightly lower values are obtained with the HK method and gives an impression of the scatter obtained, with and without the use of a final SEP-PAK. There is no obvious pattern in these data which might give clues as to the nature of impurities in individual batches. There was a similar lack of pattern in plots of TLC data versus HK data.

CONCLUSIONS

The enzymatic analytical method for 2FDG described above has several advantages over the chromatographic methods. Firstly, the assay is rapid and can be completed in 5–6 min, compared with more than 15 min for HPLC, and ~30 min for TLC. Second, the method is inexpensive. Third, the method gives results which are easy to interpret—the criteria for successful synthesis of 2FDG are minimal trapping on Dowex 1 before enzyme treatment and almost complete trapping afterwards. A fourth potential advantage is that the simplicity of the method may more easily lend itself to incorporation into automated 2FDG synthetic equipment.

A major limitation of the enzymatic method as described above is that it cannot discriminate between 2FDG and 2-deoxy-2-fluoro-D-mannose (2FDM) which is a contaminant of 2FDG prepared by electrophilic ^{18}F fluorination of glucal or triacetylglucal (*21,22*). The influence of contamination of 2FDG with 2FDM on calculated metabolic rates is not yet totally clear (*23*). Furthermore, the most commonly employed HPLC system (aminopropyl-bonded silica gel eluted with 70% aqueous acetonitrile) also cannot distinguish 2FDG from 2FDM although the two sugars may be separated under special TLC conditions (*21*). Therefore, the HK method could also be used for routine analysis of 2FDG prepared electrophilically, provided that the degree of contamination with 2FDM is known to be low. Other syntheses of 2FDG based on nucleophilic ^{18}F besides the Hammacher (*11*) method, such as those using 2,3-cyclic sulfates (*10,24*), also do not produce 2FDM. No other mono-deoxyfluoro hexoses are known or expected to be phosphorylated rapidly by HK. The known poor substrates for HK, 3FDG and 4FDG, react so slowly compared with 2FDG or 2FDM that they would not undergo significant phosphorylation with the small amount of enzyme used (*15,17*).

ACKNOWLEDGMENTS

This work was supported by the Department of Energy (DE-FG02-86ER60438) and by the National Institutes of Health (HL 36534-03). CMT was supported by an NCI Training Grant (CA 09260-06).

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