Application Note
Clinical & Diagnostic

The most selective LC-EC applications for Clinical & Diagnostics analysis

Catecholamines
Serotonin
Metanephrines
VMA
HVA
5-HIAA

PET imaging tracer
Fluorodeoxyglucose (FDG)
FDG impurities

Sulfides
Homocysteine
Glutathione
Disulfides

Vitamins, minerals
A, C, D, E, and K
Iodide
Q10, Ubiquinols

Fluorodeoxyglucose, [^{18}F]FDG, Radiotracer Analysis

- **ALEXYS Analyzer for [^{18}F]FDG and its by-products**
- **Reproducible & robust**
- **European Pharmacopoeia 8.2 (2014)**
- **U.S. Pharmacopeia 38-NF33 (2015)**

Summary

The [^{18}F]FDG analysis was evaluated on an Antec ALEXYS HPAEC-PAD analyzer using the method and conditions described in the official USP and EP monographs. The ALEXYS analyzer for [^{18}F]FDG is based on the new DECADE Elite electrochemical detector in combination with a CarboPac PA20 analytical column, which allows faster analysis of [^{18}F]FDG (compounds of interest elute within 8 minutes) and better detection of its by-products compared to previously reported data [4]. In this application note typical results obtained with the ALEXYS analyzer are reported demonstrating its suitability for the analysis of FDG in compliance with EP and USP methods.

Fluorodeoxyglucose, [^{18}F]FDG, Radiotracer Analysis

Introduction

In PET imaging, the radio-labelled tracer 2-deoxy-2-(^{18}F)fluoro-D-glucose, also called [^{18}F]-Fluorodeoxyglucose, [^{18}F]FDG or FDG, can be used for the assessment of glucose metabolism in the heart, lungs, and the brain. It is also used for imaging tumors in oncology, where usually dynamic images are analyzed in terms of Standardized Uptake Values. The 109.8 minute half-life of ^{18}F makes rapid and automated chemistry necessary; therefore [^{18}F]FDG is produced in a cyclotron in vicinity of the PET facility.

One of the tests that needs to be performed on the [^{18}F] FDG solution before it can be injected into a patient, is to check its purity. The actual concentration of the radiotracer, the by-products 2-fluoro-2-deoxy-D-mannose (FDM) and 2-chloro-2-deoxy-D-glucose (CDG) is determined. Compendial methods for this analysis are described in both the U.S Pharmacopeia (USP) and European Pharmacopoeia (EP) [1,2]. These EP and USP methods are to a large extent similar and based on Anion-Exchange Chromatography (HPAEC) in combination with Pulsed Amperometric Detection (PAD) [3].

Figure 1: ALEXYS analyzer for FDG with AS 110 autosampler.

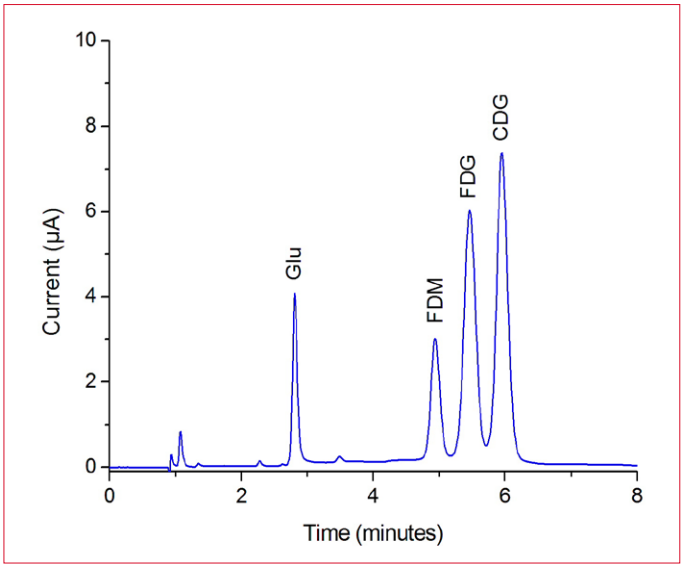

Figure 2: Chromatogram of a standard mix consisting of 25 µg/mL FDG, FDM, CDG & 2.5 µg/mL Glucose in water (20 µL injection).

Method

The EP and USP method for the analysis of FDG are based on isocratic separation using an anion exchange column and alkaline mobile phase (pH 13) followed by pulsed amperometric detection on a gold (Au) working electrode. For the method evaluation an Antec ALEXYS HPAEC-PAD analyzer consisting of an AS110 autosampler was used for automated sample analysis. In the case the number of samples per day is limited, the ALEXYS HPAEC-PAD Analyzer can optionally be configured with a manual injection valve instead of an autosampler.

Separation

Under alkaline conditions (pH > 12) carbohydrates like FDG can be separated by means of HPAEC. Carbohydrates are weak acids with pKa values ranging between 12 and 14. At high pH they will be either completely or partially ionized depending on their pKa value. In the monographs the use of the following (similar) stationary phases are described:

EP: strongly basic anion-exchange resin for chromatography (particle size 10 µm), column size: l = 0.25 m, diameter 4.0 mm.

USP: 4.0 mm x 25 cm column which contains 10 µm packing L46. L46 being defined as Polystyrene/ divinylbenzene substrate agglomerated with quaternary amine functionalized latex beads, about 9 µm to 11 µm in diameter.

However, for this application a 150 mm x 3 mm ID CarboPac PA20 anion-exchange column with 6.5 µm particle size has

been chosen for the following reasons: (1) This column shows better chromatographic performance due to the smaller particle size of the stationary phase and (2) allows faster analysis of FDG (all compounds of interest elute within 7 minutes). Although the column isn't an exact match with respect to column dimensions and particle size as described in the monographs, it falls within the maximum allowed variations for these chromatographic parameters as specified in the general chapter <621> Chromatography of the USP [5] and chapter 2.2.46. Chromatographic separation techniques of the EP [6]. In table 1 an overview of the maximum allowed variations of the relevant chromatographic parameters for both USP and EP are shown for reference.

Table 1

Maximum allowed adjustments of chromatographic parameters (EP & USP)

Adjustment	*EP*	*USP*
Column length	± 70%	See particle size
Column inner diameter	± 25%	Can be adjusted if the linear velocity is kept constant.
Column particle size*	- 50%	Particle size and/or length may be modified providing that the ratio column length/particle size (L/dp ratio) remains constant or in the range between -25% to +50%.
Flow rate	± 50%	Must be adjusted if column ID has been modified, see column inner diameter
Column temperature	± 10°C	± 10°C (isocratic elution)
Injection Volume	May be decreased, providing that detection and repeatability are satisfactory.	The injection volume can be adjusted as far as it is consistent with accepted precision, linearity, and detection limits.

*) L/dp ratio of the column specified in the USP monograph is 250 mm/10x10-3mm = 25000, for the CarboPac PA20 the L/dp ratio is 150/6.5x10-3 = 23007, which falls within the max. allowed variation of the L/dp ratio (-8%).

The mobile phase was prepared as described in the EP & USP monographs (see table 2 and 5). To minimize the introduction of carbonate ions in the mobile phase the eluents were prepared using a carbonate-free 50% w/w NaOH solution (commercially available).

The diluent was deionized water (resistivity >18 MΩ.cm) which was sonicated and sparged with Helium 5.0 prior to use. The mobile phase should be prepared in plastic bottles instead of glass. NaOH is a strong etching agent and will react with the inner glass wall resulting in the release of silicates and borates. The appropriate amount of NaOH solution was carefully pipetted into the diluent under gently stirring and Helium sparging. The bottles with mobile phase and column regeneration solution were blanketed with Helium (0.2 bar overpressure) during the analysis to minimize the build-up of carbonate ions in the mobile phase and to assure a reproducible analysis. It is advisable to prepare fresh mobile phase daily. Furthermore, if a gradual loss of retention is observed due to the slow build-up of interfering anions on the column, a column regeneration step [7] can be applied. For more details about mobile phase preparation and precautions see the application note Carbohydrates in Food [7].

Detection

For the detection of FDG and its by-products pulsed amperometric detection is mandatory. A DECADE Elite electrochemical detector is used in combination with a Flexcell™. This thin-layer flow cell has a replaceable Au working electrode (WE) and maintenance-free HyREF (Pd/H_2) reference electrode. In both the EP and USP monograph no specific potential waveform for detection is described, therefore we applied an optimized 4-step potential waveform as shown in figure 3. This particular waveform resulted in an excellent reproducibility and minimal electrode wear [7]; i.e. resulting in less flow cell maintenance and system down time. The cell current was typical about 1 – 2 µA under the specified conditions.

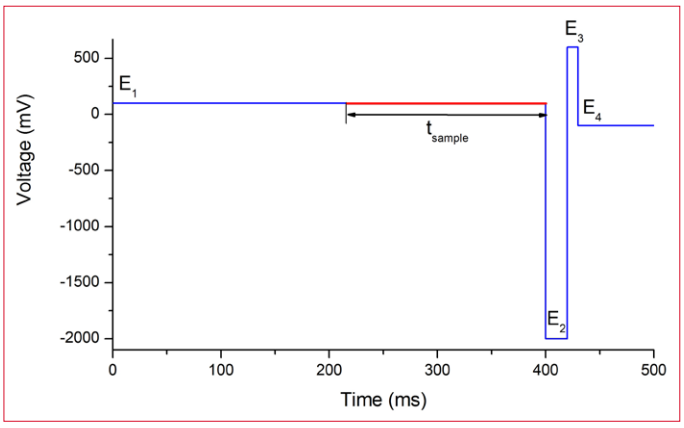

Figure 3: 4-step PAD potential waveform for the detection FDG and impurities.

The EP specifies a temperature for separation of 25°C, but allows a variation of ± 10°C [7]. For optimal performance the temperature for separation and detection was set to 30°C. In the next paragraphs the results are presented of the evaluation of the FDG analysis based on the EP and USP monographs (respectively) using the ALEXYS HPAEC-PAD analyzer.

Results - for EP monograph

The compendial method for Fludeoxyglucose F 18 injections in the EP describes a test based on HPAEC-PAD to determine:

(1) The FDG content (maximum 0.5 mg [18F]FDG per maximum recommended dose in milliliter)
(2) The amount of 'Impurity A' (CDG), 2-chloro-2-deoxy-D-glucose

The LC-EC conditions used to evaluate the EP method are listed in table 2. The flow rate was adjusted to 0.5 mL/min (50% less than specified in the monograph) to compensate for the use of a column with a smaller column internal diameter.

Table 2

LC-EC Conditions (EP)	
HPLC	ALEXYS HPAEC-PAD analyzer with AS 110 autosampler
Column	CarboPac PA20 150 x 3 mm ID analytical column + CarboPac PA20 30 x 3 mm ID guard column.
Mobile phase	4 g/L NaOH carbonate-free in water (100 mM), the mobile phase is continuous sparged with Helium 5.0
Flow rate	0.5 mL/min
Vinjection	20 µL (full loop)
Temperature	30°C for separation & detection
Flow cell	FlexCell™ with Au WE and HyREF (Pd/H_2) RE, spacer 50 µm
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
I-cell	1-2 µA
ADF	0.1 Hz
Range	10 µA/V

In figure 2 an example chromatogram is shown of a 20 µL injection of a standard mix with 6.25 µg/mL FDG, FDM, CDG & 1.5 µg/mL Glucose in water. The relative retention time for FDM, FDG and CDG (see table 3) correspond to the values indicated in the monograph.

Table 3

Retention time of FDG and related substances		
Parameter	*Retention (min)*	*Relative retention**
Glucose	2.81	0.5
FDM	4.94	0.9
FDG	5.47	1.0
CDG	5.96	1.1

**) Relative retention time (RRT) with reference to FDG (5.47 min).*

System suitability

In the EP monograph for [18F]FDG injections the following system suitability requirements are specified to evaluate the system performance:

- Resolution: between FDM and FDG ≥ 1.5
- Signal-to-noise ratio: for FDG ≥ 10

The system suitability is evaluated using the chromatogram obtained with reference solution (c), shown in figure 4. The reference solution (c) was prepared according to the guidelines given in the EP, taking into account a "maximum recommended dose, in milliliters of 20 mL". In this case, reference solution (c) has a final concentration of 25 µg/mL FDM and 12.5 µg/mL FDG (137 µM and 68 µM respectively).

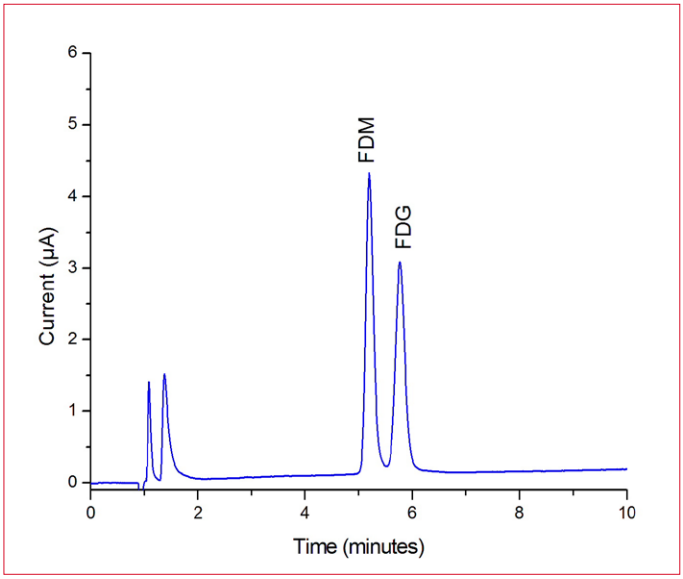

Figure 4: Chromatogram of a 25 µg/mL FDM and 12.5 µg/mL FDG in water (20 µL reference solution (c) as described in the EP monograph).

Table 4

EP system suitability requirement		
Parameter	*EP criteria*	*Measured*
Resolution between FDG & FDM	> 1.5	> 1.8
Signal-to-Noise ratio FDG	> 10	> 700

In figure 2 an example chromatogram is shown of a 20 µL injection of a standard mix with 6.25 µg/mL FDG, FDM, CDG & 1.5 µg/mL Glucose in water. The relative retention time for FDM, FDG and CDG (see table 3) correspond to the values indicated in the monograph.

Linearity, repeatability and detection limit

The linearity of FDG, FDM and CDG was investigated in the concentration range of 0.5 – 25 µg/mL (3 – 140 µM) and 0.05 – 2.5 µg/mL for Glucose (0.1 – 14 µM). The correlation coefficients were better than 0.999 for peak areas and peak heights for all compounds.

The relative standard deviation (RSD) for peak area, peak height and retention time was determined for 8 replicate injections of a standard of 25 µg/mL FDM, FDG and CDG in water. The RSD for retention time was 0.1% for all compounds and better than 2% for both peak height and peak area

Table 5

LOD – EP method (20 µL injection)			
Compound	LOD (ng/mL)	LOD (nM)	On-column (ng)
Glucose	5	30	0.1
FDM	85	465	1.7
FDG	36	200	0.7
CDG	30	150	0.6

The Limit of Detection (LOD) for Glucose, FDM, FDG and CDG are shown in table 5. The LOD's were calculated as the analyte response corresponding to 3x the ASTM noise (average peak-to-peak baseline noise of 30 segments of 0.5 min). The responses of a chromatogram obtained with a 100 ng/mL mix of standards (10 ng/mL for Glucose) were used to calculate the LOD. Concentration detection limits for FDG and related compounds were in the range of 30 – 85 ng/mL. For Glucose a detection limit of 5 ng/mL (30 nM) was measured, which corresponds with detection limit for Glucose reported in previous studies with the FlexCell. To demonstrate the good detection sensitivity of the ALEXYS HPAEC-PAD system a chromatogram of a 20 µL injection of a standard mix with 100 ng/mL FDG, FDM, CDG & 10 ng/mL Glucose in water, is shown in figure 5.

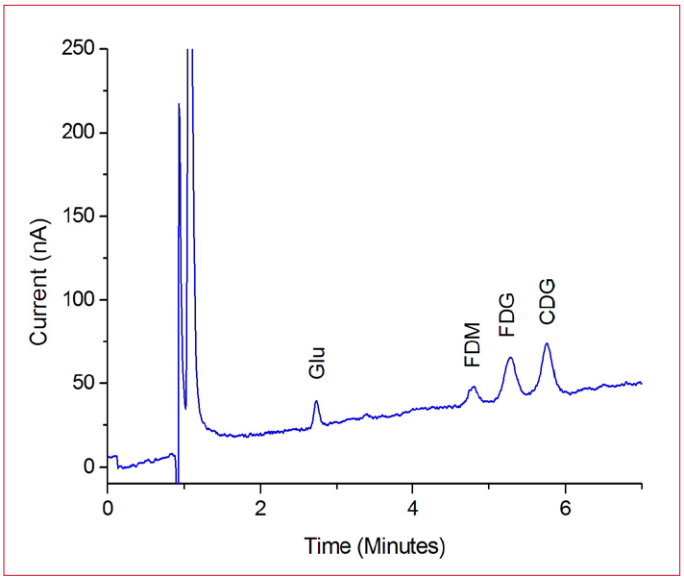

Figure 5: Chromatogram of a standard mix with 100 ng/mL FDG, FDM, CDG & 10 ng/mL Glucose (Glu) in water (20 µL injection).

Results - for USP monograph

The compendial method for Fluodeoxyglucose (^{18}F) injections in the USP describes a test based on HPAEC-PAD to determine the amount of 'Fluodeoxyglucose related compound B' (CDG), 2-chloro-2-deoxy-D-glucose. Note that in the EP, CDG is designated as 'Impurity A'.

The LC-EC conditions used to evaluate the USP method are listed in table 6. There a few differences compared to the EP conditions (table 2):

Flow rate: the USP requires a flow rate adjustment to keep the linear velocity constant in the case the column diameter is changed [5]. The exact text stated in the general chapter '<621> chromatography':

"Column inner diameter (HPLC): can be adjusted if the linear velocity is kept constant. Flow rate changes for both a change in column diameter and particle size can be made by:

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)]$$

where F1 and F2 are the flow rates for the original and modified conditions, respectively; dc1 and dc2 are the respective column diameters; and dp1 and dp2 are the particle sizes."

Based on this considerations a flow rate of 0.4 mL/min was chosen:

$$F_2 = F_1 \times [(dc_2^2 \times dp_1)/(dc_1^2 \times dp_2)] = 0.5 \times [(9 \times 10)/(16 \times 6.5)] = 0.4 \text{ mL/min}.$$

Injection volume: in case of the USP method significant higher concentrations of reference standards are injected, therefore the injection volume was lowered to 1 µL to avoid peak broadening/overloading. Adjustments of injection volume are allowed within the USP, as long as it is consistent with accepted precision, linearity and detection limits [5].

Guard column: to meet compliance no guard column was used. The USP general chromatography chapter [5] specifies: a guard column may be used with the following requirements: (a) the length of the guard column must be not more than 15% of the length of the analytical column b) the inner diameter of the guard must be the same as the analytical column and (c) the packing material should be the same as the analytical column. For the CarboPac PA20 the available guard column has the following dimensions: 30 x 3 mm ID, which exceeds the maximum length limit of < 15%.

Table 6

LC-EC Conditions (USP)	
HPLC	ALEXYS HPAEC-PAD analyzer with AS 110 autosampler
Column	CarboPac PA20 150 x 3 mm ID analytical column
Mobile phase	4 g/L NaOH in carbonate-free water (100 mM), the mobile phase is continuous sparged with Helium 5.0
Flow rate	0.4 mL/min
Vinjection	1 µL (partial loop injection)
Temperature	30°C for separation & detection
Flow cell	FlexCell™ with Au WE and HyREF (Pd/H_2) RE, spacer 50 µm
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
I-cell	1-2 µA
ADF	0.1 Hz
Range	20 µA/V

System suitability

In the USP monograph for [^{18}F]FDG injections the following system suitability requirements are specified to evaluate the system performance:

- Resolution: between FDM and FDM ≥ 1.5
- Repeatability: RSD in peak area's ≤ 5%

The system suitability requirements are evaluated using the chromatogram shown in figure 6 obtained with the system suitability solution prepared according to the guidelines given in the USP.

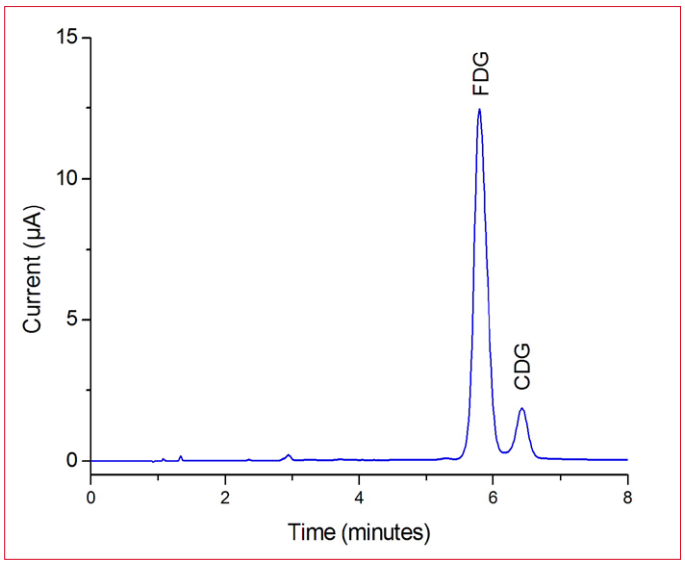

Figure 6: Chromatogram of 1.0 mg/mL FDG & 0.1 mg/mL CDG in mobile phase (1 µL injection of system suitability solution as described in the USP monograph).

Table 7

USP system suitability requirement

Parameter	*USP criteria*	*Measured*
Resolution between FDG & FDM	> 1.5	> 1.75
RSD peak area FDG, n=6	< 5%	2.0
RSD peak area CDG, n=6	< 5%	1.7

The RSD was calculated based on the data of 6 replicate injections of the system suitability solution as advised in the general chapter '<621> chromatography' [5]. The results are listed in table 7, it is evident that the system suitability requirements are easily met for all performance parameters.

Linearity and detection limit

Linearity of FDG and CDG was investigated in the concentration range of 200 – 1000 µg/mL and 20 – 100 µg/mL respectively (1 – 6 mM and 100 – 500 µM). The correlation coefficients were better than 0.999 for peak areas and peak heights for both FDG and CDG.

Table 8

LOD – USP method (1 µL injection)

Compound	*LOD (ng/mL)*	*LOD (µM)*	*On-column (ng)*
FDG	680	3.7	0.7
CDG	504	2.5	0.5

The LOD for FDG and CDG are shown in table 5. The responses obtained from a chromatogram of a 1 µL injection of a 500 ng/mL mix of standards were used to calculate the LOD. The on-column detection limits for both FDG and CFG obtained are comparable to the ones reported in table 5 for the EP method. Note that the concentration LOD's are significantly higher than the ones reported for the EP method. It is evident that this is caused by the smaller injection volume used (1 µL versus 20 µL). Even with a 1 µL injection volume the detection limit for FDG related compound B (CDG) obtained under these conditions, is well below the allowed limit of CDG (not more than 1 mg per dose) in [18F]FDG injections as specified in the USP monograph. For example: if the maximum recommended dose is 20 mL this corresponds to a maximum allowed concentration of 1 mg CDG in 20 mL = 50 µg/mL, which is a factor 100x higher in concentration than the measured LOD for CDG.

Conclusion

The ALEXYS FDG Analyzer provides an excellent solution for the analysis of FDG and its by-products in [18F]FDG injections following the compendial methods of the EP and USP.

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Ordering number

180.0053E	ALEXYS FDG Analyzer with manual injector
191.0036L	AS 110 autosampler standard 6p, LAN
250.1078	CarboPac PA20 analytical column, 150 x 3.0 mm ID
250.1079	CarboPac PA20 guard column, 50 x 3.0 mm ID

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