Appendix U Methods of 24-hour urine analysis and quality control

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U.1 Introduction

This appendix describes the methods used to analyse urinary analytes and provides details of the quality control procedures for these assays in Years 1 to 4 of the National Diet and Nutrition Survey Rolling Programme (NDNS RP). The quality of the laboratory analyses is assured by rigorous instrument maintenance, staff training, adherence to standard operating procedures and good laboratory practice. The quality control and assessment practices used at HNR are all standard procedures for the type of assay used and HNR is ISO certified (BS EN ISO 9001:2008).

U.2 Analysis of urine samples

U.2.1 Sodium and potassium

Urinary sodium and potassium have been measured using ion-specific electrodes (ISEs) during the NDNS RP. The sodium and potassium methods on the Siemens Dimension® Xpand clinical chemistry system with the QuikLYTE® module are *in vitro* diagnostic tests intended for the quantitative measurement of sodium and potassium in urine, which use indirect sample sensing with the QuikLYTE® Integrated Multi-sensor Technology (IMT) to develop an electrical potential proportional to the activity of each specific ion in the sample. Each urine sample is diluted automatically and then transferred automatically to the sensor, where Na^+ and K^+ ions establish equilibrium with the electrode surface. A potential is generated proportional to the logarithm of the analyte activity in the sample. The electrical potential generated by a sample is compared with the electrical potential generated by a standard solution, and the concentration of the desired ions is calculated. Sampling, dilution, reagent delivery, mixing, processing, calculation and printing of results are automatically performed by the Dimension® system. Samples are identified with bar codes; the instrument automatically uploads barcode and concentration information to a results spreadsheet, thus eliminating transcription errors. The assay range is for sodium 5–300mmol/L and for potassium 1–300mmol/L.

U.2.1.1 Quality controls for sodium and potassium

U.2.1.1.1 Internal quality controls

Internal commercially-prepared quality control samples (QCs; Biorad Liquichek, Level 1 and Level 2) are run on the analyser to check for correct calibration and function before the samples are analysed, and included in every batch to determine between-assay precision. Once a bottle is opened, the remaining volume is aliquoted into smaller tubes and frozen at -20°C. QC material is brought to room temperature and mixed thoroughly before use. The result obtained should fall within limits defined

by the day-to-day variability of the system as measured in the laboratory. The results are recorded (mean, sd, %cv) and for each QC it is checked that the result obtained is within the manufacturer's specified range and also within the more stringent range determined within the laboratory. The QC results demonstrate good precision throughout Years 1 to 4 of the RP.

Table U.1 Year 1 QC data for sodium assay

	Lvl 1 lot 62871	Lvl 2 lot 62872
N	11	11
Mean	78.9	160
sd	1.54	3.36
%cv	1.95	2.10

Table U.2 Year 2 QC data for sodium assay

	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	28	8	28	8
Mean	79.0	77.2	161	163
sd	1.36	0.46	2.07	1.04
%cv	1.72	0.60	1.29	0.64

Table U.3 Year 3 QC data for sodium assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	35	35
Mean	76.4	162
sd	1.09	3.14
%cv	1.43	1.93

Table U.4 Year 4 QC data for sodium assay

Lvl 1 lot 63221	Lvl 1 lot 63221	Lvl 2 lot 63222
N	20	20
Mean	76.3	161
sd	1.20	2.43
%cv	1.57	1.51

Table U.5 Year 1 QC data for potassium assay

	Lvl 1 lot 62871	Lvl 2 lot 62872
N	11	11
Mean	27.8	61.1
sd	0.39	0.99
%cv	1.39	1.62

Table U.6 Year 2 QC data for potassium assay

Lvl 1 lot 62871	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	28	8	28	8
Mean	28.2	29.2	62.3	64.9
sd	0.521	0.514	1.11	1.33
%cv	1.84	1.76	1.78	2.04

Table U.7 Year 3 QC data for potassium assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	35	35
Mean	29.7	67.5
sd	0.73	3.28
%cv	2.45	4.86

Table U.8 Year 4 QC data for potassium assay

Lvl 1 lot 63221	Lvl 1 lot 63221	Lvl 2 lot 63222
N	20	20
Mean	31.2	69.9
sd	2.22	5.51
%cv	7.11	7.89

U.2.1.1.2 External quality assessment

The HNR Nutritional Biomarker Analysis Laboratory is a member of NEQAS (National External Quality Assessment Scheme) for urinary sodium and potassium - this scheme sends samples to all hospital and many other analytical laboratories in the UK for analysis and compares results, to improve harmonization of results between laboratories.

NEQAS controls were run concurrently with NDNS urines. NEQAS samples are urine or artificial matrices spiked to simulate the range of concentrations found in human urine.

Tables U.9 and U.10 summarise the results obtained using the HNR Siemens Dimension assay relative to those obtained by other laboratories using the same method, and relative to the All Laboratories Trimmed Mean (ALTM) derived from the results of all participating laboratories by combining results obtained by all methods. Consistency of HNR results is demonstrated across the four years of the NDNS RP.

Table U.9 NEQAS results for urinary sodium analyses (Years 1 to 4)

NEQAS	Year 1	Year 2	Year 3	Year 4
% bias from method mean	-4.9	-1.6	-4.2	-0.7
SD of bias	2.7	1.9	3.4	2.1
% bias from ALTM	-5.9	-3.5	-6.1	-5.3
SD of bias	1.7	2.8	2.2	1.2

Table U.10 NEQAS results for urinary potassium analyses (Years 1 to 4)

NEQAS	Year 1	Year 2	Year 3	Year 4
% bias from method mean	0.2	2.9	1.4	5.8
SD of bias	3.1	3.0	1.8	3.6
% bias from ALTM	-3.6	-3.8	-4.0	1.0
SD of bias	2.4	3.0	2.0	1.6

U.2.2 Creatinine

The creatinine method employs a modification of the kinetic Jaffe reaction (Larsen). In the presence of a strong base such as sodium hydroxide, picrate reacts with creatinine to form a red chromophore. The rate at which absorbance at 510nm increases due to the formation of the chromophore is directly proportional to the creatinine concentration in the sample and is measured using a bichromatic (510, 600nm) rate technique. Bilirubin is oxidised by ferric ferricyanide to prevent interference.

$$\text{Creatinine + picrate} \xrightarrow{\text{NaOH}} \text{red chromophore (absorbs at 510nm)}$$

Creatinine in boric acid treated urine is stable for two to three days at room temperature and therefore posting of the urine aliquots to the laboratory was acceptable.

The assay range is 0 – 1768µmol/L. The limit of detection of the creatinine method is 4µmol/L; this represents the lowest concentration of creatinine that can be statistically distinguished from zero (Siemens data).

Creatinine excretion is affected by muscle mass and therefore varies considerably from person to person.

U.2.2.1 Quality controls for Creatinine

U.2.2.1.1 Internal quality controls

The creatinine assay on the Siemens Dimension® Xpand is controlled with Lyphochek QC 1 and 2 produced by Bio-Rad Laboratories, included in every batch to determine between-batch precision. Once a bottle is opened, the remaining volume is aliquoted out into smaller tubes and frozen at -20°C. QC material is brought to room temperature and mixed properly before use. The result obtained should fall within limits defined by the manufacturer and also within the more stringent range defined by the laboratory.

Table U.11 Year 1 QC data for creatinine assay

	Lvl 1 lot 62871	Lvl 2 lot 62872
N	11	11
Mean	5.36	12.24
sd	0.17	0.42
%cv	3.16	3.43

Table U.12 Year 2 QC data for creatinine assay

	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	30	8	30	8
Mean	5.33	5.87	11.9	12.7
sd	0.11	0.12	0.27	0.19
%cv	2.05	2.05	2.31	1.48

Table U.13 Year 3 QC data for creatinine assay

Lvl 1 lot 63221	Lvl 1 lot 63221	Lvl 2 lot 63222
N	45	45
Mean	5.68	12.0
sd	0.18	0.40
%cv	3.26	3.29

Table U.14 Year 4 QC data for creatinine assay

Lvl 1 lot 63221	Lvl 1 lot 63221	Lvl 2 lot 63222
N	30	30

Mean	5.27	11.2
sd	0.116	0.256
%cv	2.20	2.27

U.2.2.1.2 External quality assessment

HNR subscribes to NEQAS for urinary creatinine; this scheme sends samples to all hospital and many other analytical laboratories in the UK for analysis and compares results, to improve harmonization of results between laboratories.

NEQAS controls were run concurrently with NDNS urines. NEQAS samples are urine or artificial matrices spiked to simulate the range of concentrations found in human urine.

Table U.15 summarises the results obtained using the HNR Siemens Dimension assay relative to those obtained by other laboratories using the Siemens Dimension kinetic Jaffe method, and relative to the All Laboratories Trimmed Mean (ALTM) derived from the results of all participating laboratories.

Table U.15 NEQAS results for urinary creatinine analyses (Years 1 to 4)

	Year 1	Year 2	Year 3	Year 4
% bias from method mean	-8.7	-5.5	-3.5	-6.4
SD of bias	1.9	2.3	2.8	2.8
% bias from ALTM	-7.3	-2.6	-3.3	-2.5
SD of bias	2.4	3.3	3.8	1.7

U.2.3 Urea

The urea method employs a urease/glutamate dehydrogenase coupled enzymatic technique. Urease specifically hydrolyzes urea to form ammonia and carbon dioxide. The ammonia is used by the enzyme glutamate dehydrogenase (GLDH) to reductively aminate α-ketoglutarate (α-KG), with simultaneous oxidation of reduced nicotinamide-adenine dinucleotide (NADH). The change in absorbance at 340nm due to the disappearance of NADH is directly proportional to the urea concentration in the sample and is measured using a bichromatic (340, 383nm) rate technique.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_3 + CO_2$$

$$NH_3 + \alpha\text{-KG} + NADH \xrightarrow{GLDH} \text{L-glutamate} + NAD$$

U.2.3.1 Quality controls for Urea

U.2.3.1.1 Internal quality controls

The urea assay on the Siemens® Dimension Xpand is controlled with Lyphochek QC 1 and 2, produced by Bio-Rad Laboratories, included in every assay run to determine between-batch precision. Once a bottle is opened, the remaining volume is aliquoted out into smaller tubes and frozen at -20°C. QC material is brought to ambient temperature and mixed before use. The result obtained should fall within limits defined by the supplier and within the more stringent range defined by the laboratory.

Table U.16 Year 1 inter-assay variation for urinary urea

	Lvl 1 lot 62871	Lvl 2 lot 62872
N	11	11
Mean	172	288
sd	3.98	5.38
%cv	2.32	1.87

“Lyphochek 1” and “Lyphochek 2” were assayed before and with each run to check instrument performance and calibration.

Table U.17 Year 2 inter-assay variation for urinary urea

	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	21	8	21	8
Mean	170	164	283	282
sd	4.01	3.77	6.58	10.8
%cv	2.36	2.29	2.33	3.84

Table U.18 Year 3 inter-assay variation for urinary urea

Lvl 1 lot 63221	Lvl 1 lot 63221	Lvl 2 lot 63222
N	28	28
Mean	167.5	285
sd	3.82	5.97
%cv	2.28	2.10

Table U.19 Year 4 inter-assay variation for urinary urea

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	14	14
Mean	160	270
sd	7.12	13.9
%cv	4.44	5.159

U.2.3.1.2 External quality assessment

HNR subscribes to NEQAS for urinary urea; this scheme sends samples to all hospital and many other analytical laboratories in the UK for analysis and compares results, to improve harmonization of results between laboratories.

NEQAS controls were run concurrently with NDNS urines. NEQAS samples are urine or artificial matrices spiked to simulate the range of concentrations found in human urine.

Table U.20 summarises the results obtained using the HNR Siemens Dimension assay relative to those obtained by other laboratories using the Siemens Dimension kinetic Jaffe method, and relative to the All Laboratories Trimmed Mean (ALTM) derived from the results of all participating laboratories.

Table U.20 NEQAS results for urinary urea analyses (Years 1 to 4)

NEQAS	Year 1	Year 2*	Year 3	Year 4
% bias from method mean	-1.4	-4.5	-4.7	-7.8
SD of bias	4.6	2.9	3.3	4.1
% bias from ALTM	-1.7	-4.5	1.5	-0.9
SD of bias	6.9	3.7	2.4	5.9

* 1 gross outlier omitted

U.2.4 Nitrogen

Twenty-four hour nitrogen excretion has been used to validate protein intake estimated by various dietary record methods.

The NDNS RP is the first time urinary nitrogen has been measured in NDNS and the assay was subcontracted to the Institute of Grassland and Environmental Research (IGER), now the Institute for Biological, Environmental and Rural Sciences (IBERS), at the University of Aberystwyth, UK. The laboratory is accredited to ISO 17025.

Urinary nitrogen is determined using the FP-428 LECO Nitrogen Determinator. Encapsulated samples of approximately 200mg weight are combusted in high purity oxygen. The combustion products, mainly CO_2, H_2O, NO_x and N_2, are passed through a thermoelectric cooler to remove most of the water.

A 10 cm^3 aliquot of the gas is swept through a hot copper catalyst to change NO_x to N_2, then through magnesium perchlorate and sodium hydroxide to remove CO_2 and H_2O respectively.

The remaining product, N_2, is measured by a thermal conductivity cell.

U.2.4.1 Quality controls for Nitrogen

U.2.4.1.1 Internal quality controls

Two (Year 1 to Year 3) or three (Year 4) controls were used to determine between-batch precision.

Table U.21 Internal quality controls for urinary nitrogen

	Year 1		Year 2		Year 3		Year 4		
	1	2	1	2	1	2	1	2	3
Mean (%N)	9.54	1.69	9.58	1.70	9.64	1.72	9.61	1.70	1.00
SD	0.046	0.035	0.033	0.023	0.033	0.028	0.031	0.035	0.045
CV %	0.63	2.08	0.34	1.35	0.34	1.63	0.33	2.05	4.51
n	732	835	82	75	92	89	185	204	57

U.2.4.1.2 External quality assessment

There is no external quality assessment scheme available for urinary nitrogen analysis.

U.2.5 PABA (*para* aminobenzoic acid)

In Years 1 and 2, all samples were initially analysed using the conventional colorimetric procedure. This assay is subject to interference from substances containing a primary aromatic amine, such as paracetamol. In such urines the

apparent PABA concentration may be greater than 100% of the dose. Therefore, following the protocol of Bingham et al,[2] where the colorimetric assay indicated that >119% of the dose had been recovered in the urine the sample was re-analysed by a more specific HPLC method, which is unaffected by interfering substances. Approximately 20% of urines analysed during Years 1 and 2 were reassayed by HPLC for this reason. For Years 3 and 4 all samples were analysed by the specific HPLC method only, in order to better standardise interpretation.

U.2.5.1 PABA Analysis by colorimetry

The basis of the assay for analysis of PABA in 24-hour urine is the Bratton and Marshall[1] diazocoupling reaction. The method was adopted by Bingham and Cummings[2] and HNR use this version of the method, adapted to a microplate format. The amine group of the PABA reacts with nitrous acid to form a diazonium salt. Excess nitrite is removed with sulphamate to prevent nitrosamine formation. The diazonium salt is then coupled with a colour reagent and the stable dye thus formed can be measured colorimetrically and the absorbance at 530nm interpolated from a calibration curve.

In order to prepare urines for the above procedure an alkaline hydrolysis step is performed. This is required because PABA metabolites, formed as PABA passes through the liver, may be acetylated on the amine group and therefore unavailable for diazonium salt formation. A two-hour hydrolysis with sodium hydroxide converts the metabolites back to the free molecule. The assay was performed in duplicate and if duplicates differed by more than 10% the sample was reanalysed.

The reference range for PABA excretion as assessed by the colorimetric assay indicating a complete 24-hour urine collection was confirmed at HNR as 85-119% of the 240 mg dose, using 50 adult volunteers (unpublished data).

Comparison at HNR of the PABA excretion kinetics in adults and children after taking a single tablet indicated that there is no reason to expect the range indicating a complete collection to be different in children aged 4 to 10 years (unpublished data).

PABA excretion may be lower in elderly subjects.[3]

U.2.5.2 PABA Analysis by HPLC

The traditional colorimetric method may overestimate PABA in some urine collections as it cannot discriminate between PABA and primary aromatic amines that may originate from drugs, most commonly paracetamol.[2]

PABA metabolites in urine are hydrolysed under alkaline conditions, the solution is then neutralised and the resultant PABA determined by HPLC. The HPLC method is a reverse–phase method using an internal standard to compensate for volume

losses during hydrolysis. The PABA HPLC method used at HNR is based upon that previously used at the MRC Dunn Nutrition Unit which in turn is based upon the method described by Jakobsen *et al*., (1997).[4] The PABA HPLC method was then modified at HNR to replace the acetonitrile in the mobile phase with methanol because of the unavailability of acetonitrile.

Completeness of hydrolysis is monitored by including a sample containing PAHA (para amino hippuric acid) with each batch. This is hydrolysed to PABA which is then quantitated by HPLC.

The reference range for PABA excretion as assessed by the HPLC assay indicating a complete 24 hour urine collection was confirmed at HNR as 70-104% of the 240 mg dose, using 50 adult volunteers (mean ±2 sd range).

Comparison of the PABA excretion kinetics in adults and children after taking a single tablet indicated that there is no reason to expect the range indicating a complete collection to be different in children aged 4 to 10 years.

PABA excretion may be lower in elderly subjects.[3]

U.2.5.3 Quality controls for PABA by colorimetry

U.2.5.3.1 Internal quality control

A 24-hour urine collection from a volunteer who had taken 3 x 80 mg PABA tablets was stored as single-use aliquots and frozen at -80^{o}C. It was assayed with every sample set (i.e. on each microplate) during the period when urines from Years 1 and 2 were being assayed in order to determine between-batch precision.

Table U.22 PABA controls during Year 1

In-house QA	PABA mg/litre
N	148
Mean	98.2
sd	13.8
%cv	14.0

Table U.23 PABA controls during Year 2

In-house QA	PABA mg/litre
N	226
Mean	95.3
sd	15.3
%cv	16.1

No colorimetric PABA assays were performed during Year 3 or Year 4.

U.2.5.4 Quality controls for PABA by HPLC

U.2.5.4.1 Internal quality control

A sample of urine containing PABA is analysed with each batch of samples in order to determine inter-assay variation. Assay results for each run are accepted if the QC results fall within limits defined within the laboratory, otherwise the batch is re-assayed. Completeness of hydrolysis is monitored by including a sample containing PAHA (para amino hippuric acid) with each batch. This is hydrolysed to PABA which is then quantitated by HPLC. 2mM PAHA theoretically yields PABA 2mM (i.e. 275 mg/litre).

Table U.24 PABA controls during Year 1

	PABA mg/litre	PAHA hydrolysis control
N	60	29
Mean	74.7	253
sd	6.88	11.5
%cv	9.21	4.55

Table U.25 PABA controls during Year 2

	PABA mg/litre	PAHA hydrolysis control
N	44	44
Mean	73.1	258
sd	5.88	10.0
%cv	8.05	3.88

Table U.26 PABA controls during Year 3

	PABA mg/litre	PAHA hydrolysis control
N	57	58
Mean	76.6	259
sd	6.98	8.72
%cv	9.11	3.37

Table U.27 PABA controls during Year 4

	PABA mg/litre	PAHA hydrolysis control
N	52	51
Mean	72.5	265
sd	7.15	11.0
%cv	9.86	4.15

U.2.5.4.2 External quality assessment – colorimetric and HPLC assays

There is no external quality assessment scheme for PABA.

[1] Bratton AC, Marshall GK. A new coupling component for sulfanilaxnide determination. *J* Biol Chem 1939;128:537-50.

[2] Bingham S and Cummings J H. The use of 4-aminobenzoic acid as a marker to validate the completeness of 24h urine collections in man. Clin Sci (Lond) 1983; 64(6):629-35.

[3] Leclercq C, Maiani G, Polito A, Ferro-Luzzi A. Use of PABA test to check completeness of 24-h urine collections in elderly subjects. Nutrition 1991; 7: 350-354.

[4] Jakobsen J, Ovesen L, Fagt S, et al. Para-aminobenzoic acid used as a marker for completeness of 24-hour urine: Assessment of control limits for a specific HPLC method. Eur J Clin Nutr 1997; 5: 514.