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Short Communication

Automation of a high-performance liquid chromatographic assay for the determination of nicotine, cotinine and 3-hydroxycotinine in human urine*

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Introduction

The determination of nicotine and its principal urinary metabolites cotinine and 3-hydroxycotinine in urine is normally achieved using multiple gas chromatographic assay methods [1, 2].

A simple colorimetric-HPLC assay was evaluated [3, 4], in which coloured complexes of the analytes were produced with diethylthiobarbituric acid. The method was shown to have sufficient sensitivity to support studies into the metabolism of nicotine, but was unsuitable as a routine assay because of the instability of the coloured complexes [5]. Attempts to stabilize the complexes have been only partially successful in our laboratory.

Studies into the degradation of the analyte complexes with time suggested that automation of sample preparation would limit the effect of the instability by standardization of the time between reagent addition and sample analysis. A Waters Milli-Lab system was evaluated for the automation of the sample pretreatment and automatic injection onto the HPLC system.

Experimental

Materials

Nicotine hydrogen tartrate was obtained from BDH Ltd (Blaydon-on Tyne, Tyne and Wear, UK). Cotinine-free base was obtained from Sigma Chemicals (Fancy Road, Poole, Dorset, UK). *N*-acetyl nornicotine and 3-hydroxycotinine free base were supplied by Rothmans International Services Ltd (Nevendon Road, Basildon, Essex, UK). Diethylthiobarbituric acid and 1-heptane sulphonic acid sodium salt were obtained from Aldrich Chemicals Ltd (New Road, Gillingham, Kent, UK). Potassium cyanide, chloramine-T, and water were obtained from BDH Ltd. Methanol was obtained from Rathburn Chemicals Ltd (Walkerburn, Peebleshire, UK). Sep-Pak C_{18} solid-phase extraction cartridges were manufactured by Millipore (Bedford, MA, USA). All reagents were Analar grade and all solvents HPLC grade.

Calibration curves

Calibration curves were prepared by diluting aqueous stock solutions of nicotine, cotinine

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and 3-hydroxycotinine (nominally 1 mg ml^{-1}) with water to give a common aqueous stock solution (20 μg ml^{-1}). Aliquots of this solution were spiked into pre-screened control human urine in round-bottomed centrifuge tubes to give calibration standards at nominal concentrations of 2000, 1000, 500, 100 and 50 ng ml^{-1} for each analyte. An aliquot (1 ml) of control human urine was used as 0 ng ml^{-1} calibration standard.

Quality control samples

Quality control samples were prepared at nominal concentrations of 100 and 1000 ng ml^{-1} for each analyte in the same manner from a duplicate set of standard solutions.

Internal standard

N-acetyl nornicotine solution (100 μl, nominally 10 μg ml^{-1}) was added to each tube prior to loading onto the Milli-Lab.

Automated sample preparation

The sample tubes containing the urine calibration standards and quality control were transferred to the Milli-Lab and the programme started.

Potassium cyanide (200 μl, 10% solution in water) was added. Precisely 15 s later chloramine-T (200 μl, 10% solution in water) was added, followed 15 s later by diethylthiobarbituric acid (1 ml, 1% solution in acetone). The tubes were then incubated for 20 min *in situ* at room temperature. During incubation a C_{18} Sep-Pak cartridge was conditioned with methanol (1 ml) and water (3.5 ml). On completion of the incubation, an aliquot (1 ml) of the crude complex was passed through the cartridge to waste. The red complex was then eluted from the cartridge with methanol (0.5 ml) into a second round-bottomed centrifuge tube and an aliquot (100 μl) injected onto the HPLC system.

Chromatography

The analytical system used for the assay comprised a Waters Milli-Lab chromatography workstation, two Jasco 880-PU pumps with gradient elution programming, and a Kratos 773 UV–vis detector set at 530 nm.

Resolution of the three analytes and the internal standard was achieved using gradient elution on a Spherisorb ODS-2 15 cm × 4.6 mm i.d. 5 μm column. The gradient consisted of an initial isocratic elution for 5 min at methanol–aqueous 0.005 M 1-heptane sulphonic acid (40:60%, v/v) after which the methanol fraction was raised by 1% per min to 60% methanol, then by 2% per min to 90% methanol. The mobile phase was then returned to 40% methanol and equilibrated for 10 min prior to injection of the next sample.

The flow rate was 1.5 ml min^{-1}. A typical chromatogram is presented in Fig. 1.

Validation

Three calibration curves in the nominal

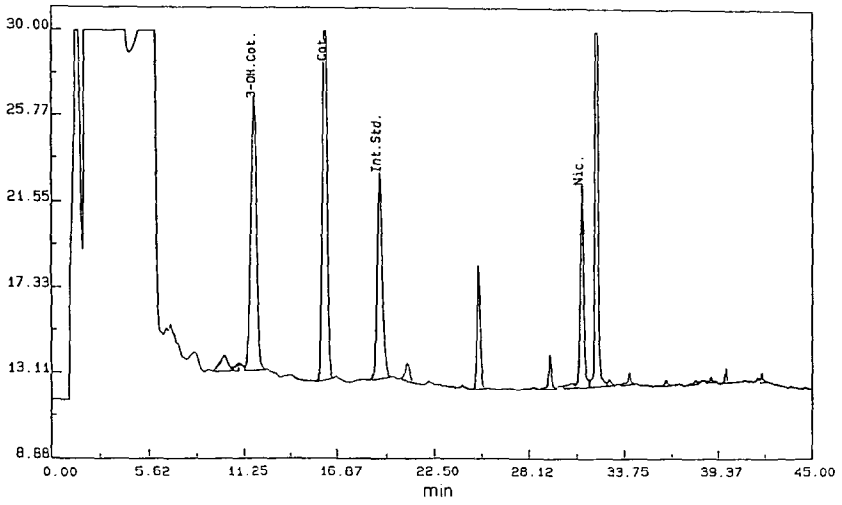

Figure 1
Resolution of nicotine and its principal urinary metabolites. Column: 15 cm × 4.6 mm 5 μm Spherisorb ODS-2. Elution: initial conditions, 40% methanol, 60% 0.005 M sodium-1 heptane sulphonate, hold 5 min; 1% per min to 60% methanol; 2% per min to 90% methanol. Detection: 530 nm.

concentration range 0–2000 ng ml^{-1} for each analyte were assayed on a single day. Quality controls were assayed in triplicate at nominal concentrations of 100 and 1000 ng ml^{-1}. Results were calculated using unweighted least-squares linear regression analysis of the peak height ratio of each analyte to the internal standard.

Results and Discussion

For nicotine, the intra-day variation ranged between 15.2–2.4% at nominal concentrations of 100 and 2000 ng ml^{-1}, respectively. The calibration curve gradients (m) were in the range 1.8784×10^{-3} to 1.9755×10^{-3}, with a relative standard deviation (RSD) of ±2.6%. Calibration curves were linear with correlation coefficients (r) of >0.999. Table 1 contains a summary of calibration data for each analyte.

For cotinine intra-day variation ranged between 1.8% at a nominal concentration of 100 ng ml^{-1}, and 1.3% at nominal 2000 ng ml^{-1}. The calibration curve gradients (m) were in the range 8.1064×10^{-4} to 8.2561×10^{-4} with a RSD of ±0.9%. Calibration curves were linear with correlation coefficients (r) of >0.999.

For 3-hydroxycotinine intra-day variation ranged between 4.5% at a nominal concentration of 1000 ng ml^{-1}, and 1.3% at nominal 2000 ng ml^{-1}. The calibration curve gradients (m) were in the range 5.3413×10^{-4} to 5.5272×10^{-4} with a relative standard deviation of ±1.7%. Calibration curves were linear with correlation coefficients (r) of >0.999.

Table 2 contains quality control data which show the method to be both accurate and precise. Precision (RSD) was <±3% for all analytes at nominal concentrations of 100 and 1000 ng ml^{-1}. Mean accuracy (% theoretical) was within 7% of nominal for all analytes at both quality control concentrations. The limit of quantification was 50 ng ml^{-1} for each analyte.

Automation of the method was effective in controlling the degradation of the analyte complexes since each sample has an identical history. Additionally, reproducibility of mixing and transfer steps controlled variation in analyte recovery. Control of the speed of sample and solvent flow through the solid-phase extraction cartridge minimized variability in analyte recovery.

Conclusion

Automation of the sample pretreatment and direct injection onto the HPLC system was effective in limiting the effect of analyte instability by standardization of the time between formation of the complex and injection onto the HPLC.

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Table 1
Calibration parameters

Analyte	n	Gradient (m)	Intercept (c)	Correlation coefficient (r)
Nicotine	3	1.8784×10^{-3} to 1.9755×10^{-3}	4.3943×10^{-2} to 9.5232×10^{-4}	0.9996 to 0.9999
Cotinine	3	8.1064×10^{-4} to 8.2561×10^{-4}	2.1241×10^{-3} to 8.6595×10^{-3}	0.9998 to 0.9999
3-Hydroxycotinine	3	5.3413×10^{-4} to 5.5272×10^{-4}	1.0493×10^{-3} to 4.3542×10^{-3}	0.9998 to 1.0000

Calibration function $y = mx + c$.

Table 2
Quality control data

Quality control conc. (ng ml^{-1})	Nicotine found (ng ml^{-1})	Cotinine found (ng ml^{-1})	3-Hydroxycotinine found
100	108	105	106
	103	103	104
	108	103	106
1000	986	1027	992
	1001	1036	997
	996	1015	976

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