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Development and validation of a dried blood spot—LC–APCI–MS assay for estimation of canrenone in paediatric samples

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ABSTRACT

A selective and sensitive liquid chromatography (LC)–atmospheric pressure chemical ionisation (APCI)–mass spectroscopic (MS) assay of canrenone has been developed and validated employing Dried Blood Spots (DBS) as the sample collection medium. DBS samples were prepared by applying 30 μl of spiked whole blood onto Guthrie cards. A 6 mm disc was punched from the each DBS and extracted with 2 ml of methanolic solution of 17α-methyltestosterone (Internal Standard). The methanolic extract was evaporated to dryness and reconstituted in acetonitrile:water (1:9, v/v). The reconstituted solution was further subjected to solid phase extraction using HLB cartridges. Chromatographic separation was achieved using Waters Sunfire C18 reversed-phase column using isocratic elution, followed by a high organic wash to clear late eluting/highly retained components. The mobile phase consisted of methanol:water (60:40, v/v) pumped at a flow rate of 0.3 ml/min. LC–APCI–MS detection was performed in the selected-ion monitoring (SIM) mode using target ions at *m/z* 341.1 and 303.3 for canrenone and internal standard respectively. The selectivity of the method was established by analysing DBS samples from 6 different sources (individuals). The calibration curve for canrenone was found to be linear over 25–1000 ng/ml ($r > 0.994$). Accuracy (% RE) and precision (% CV) values for within and between day were <20% at the lower limit of quantification (LLQC) and <15% at all other concentrations tested. The LLOQ of the method was validated at 25 ng/ml. Clinical validation of the method was achieved by employing the validated method for analysis of 160 DBS samples from 37 neonatal and paediatric patients.

1. Introduction

Diuretics are one of the most commonly used medications in hospitalised paediatric patients. They are used to promote the secretion of water and electrolytes in states of fluid overload associated with a range of acute and chronic clinical disorders including pulmonary oedema, congestive cardiac failure and hypertension [1]. Spironolactone (Fig. 1), a aldosterone antagonist, is the diuretic of choice in paediatric patients [2], however its parenteral use is limited because of its low solubility. Potassium canrenoate (Fig. 1) is a water soluble steroidal diuretic structurally related to spironolactone and is used as an injectable formulation with therapeutic activity similar to that of spironolactone [3].

Potassium canrenoate is not licensed for use in children or neonates in the UK but it is used frequently as a short-term parenteral treatment in paediatric patients for whom oral treatment with spironolactone is difficult or impossible [4]. Potassium canrenoate is rapidly converted to canrenone (CAN; Fig. 1), an active metabolite after administration [5]. CAN has been shown to have 10 times the affinity for the mineralocortecoid receptors when compared with potassium canrenoate due to the necessity of the closed lactone ring for activity [6].

Despite the frequency of their use in paediatric patients, there have been no published pharmacokinetic (PK) studies and very few studies to document the efficacy and safety of potassium canrenoate in the paediatric population [7]. Consequently, with the emergence of the new paediatric regulations around the world and in an effort to stimulate new research, spironolactone/potassium canrenoate have been identified as drugs for which paediatric studies are required. In 2003, it was placed on the US National Institute of Health's list of drugs requiring paediatric investigation [8]. In 2007, it was also included in The European Medicines Agency's (EMEA) priority list of off-patent medicines that require assessment in paediatric patients, noting that data on pharmacokinetics, on long term safety in all the paediatric age groups and on the safety and efficacy in premature infants were required [9].

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The sensitive and selective bioanalytical method discussed herein is part of ongoing research programme which involves the population pharmacokinetic (PK) study of medicines (used in an off-label or unlicensed manner) in neonates and children using the sparse data analysis technique and employing Dried Blood Spots (DBS) as the sample collection method. The latter technique is particularly useful in neonates and infants, in whom, for clinical and ethical reasons very low volume sampling is required.

Several HPLC methods have been reported for estimation of CAN in various biomatrices such as serum, plasma and urine [10–26], however there are no published methods which have employed whole blood in liquid form or in the form of DBS. Many of the methods have several limitations such as lack of selectivity [10,11], low sensitivity [13,17] or large volume of biomatrix required (1 ml plasma) [18,25] and as such are not suitable for estimation of CAN in very low volume paediatric blood samples. We have previously reported a sensitive HPLC method for estimation of spironolactone and its metabolites, including CAN, in paediatric plasma [24] employing 200 μl plasma samples however, the same method cannot be extrapolated to DBS samples as the volume of biomatrix to be processed is approximately 20 times lower than that utilised for plasma.

The collection of DBS samples on absorbant paper was first reported by Guthrie and Susi in 1960 for estimation of phenylalanine in newborns for the detection of phenylketonuria [27]. The use of DBS samples in the bioanalysis of drugs and metabolites has been reported for only a few drugs and its application in neonatal PK studies is not well explored [28–30]. The DBS technique in neonatal PK studies offers several advantages compared to conventional venipuncture such as relatively non-invasive, simple to perform, requires minimal training, enables room temperature transport/storage since most analytes are stabilised in a dry matrix, easy transportation and flexibility in collection of blood samples off-site [31–33].

The objective of the present study was to develop and validate a sensitive and selective bioanalytical method for the estimation of CAN in DBS samples suitable for use with paediatric and neonatal samples. The paper also discusses the application of the developed method in the analysis of CAN in a population PK study of potassium canrenoate in neonates and children.

2. Experimental

2.1. Chemicals and materials

Canrenone was purchased from Betapharma (Shanghai, China). 17α-Methyltestosterone (MT) was purchased from Sigma (Poole, UK). HPLC grade methanol and acetonitrile were supplied by Fisher Scientific (Loughborough, UK). HPLC grade water was obtained using a Millipore Direct-Q™ 5 Water System (Millipore, Watford, UK). Oasis® HLB solid phase extraction cartridges (1 cm^3/30 mg) were purchased from Waters (Dublin, Ireland). Guthrie cards (Schleicher & Schuell 903®) were purchased from Aston Ltd. (Oldham, England). Drug-free blood, with EDTA as anticoagulant, was obtained from healthy human volunteers. All other reagents were of analytical grade except where otherwise stated.

2.2. Preparation of stock solutions, calibration standards and quality control (QC) samples

Approximately 10 mg of CAN and MT (used as internal standard (IS)) were separately weighed and dissolved in 10 ml of methanol in a volumetric flask to give a 1 mg/ml master stock solution. Working standards of CAN were prepared by appropriate dilutions of the master stock solution with methanol. These working standards were used for the preparation of whole blood calibration standards and for the preparation of analytical standards. A MT working standard was also prepared by diluting the master stock in methanol and used as required. All stock solutions and working standards were stored in the dark at −20 °C until required.

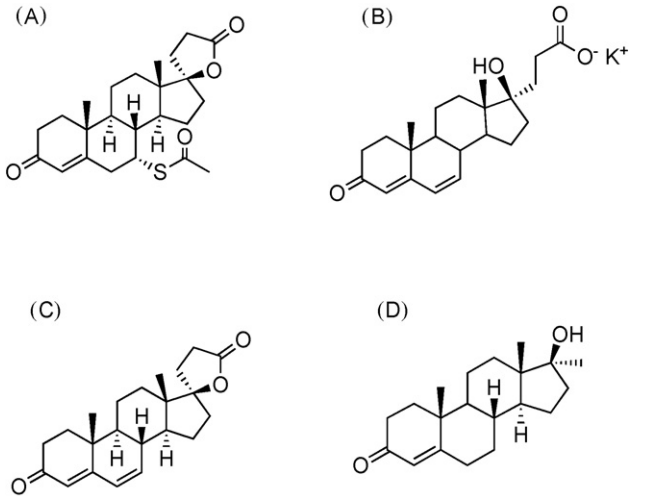

Fig. 1. Structures of spironolactone (A), potassium canrenoate (B), canrenone (C) and 17α-methyltestosterone (D).

Analytical standards (range 25–1000 ng/ml) were prepared by further dilutions of the working standard solutions in mixture of methanol:water (60:40, v/v). These analytical standards were prepared to test the linearity of the method and to estimate the recovery and the matrix effect.

The whole blood calibration standards (CS) were prepared by spiking 980 μl of blank whole blood with 20 μl of the appropriate working standard solution of CAN to produce final concentrations of 25, 50, 75, 100, 250, 500, 750 and 1000 ng/ml. The QC samples at concentrations 25 (LLQC), 50 (LQC), 250 (MQC) and 1000 (HQC) ng/ml were prepared in a similar fashion. Spiked blood was kept for 30 min at room temperature before spotting onto the Guthrie cards to allow for equilibrium and even distribution of CAN in the sample.

2.3. Blood spotting

The DBSs were prepared by spotting 30 μl of the respective whole blood standard onto Guthrie cards using a calibrated pipette. The samples were left to dry at room temperature in the dark for at least 3 h before storing at −20 °C until analysis. CS and QC samples thus prepared were used in the validation of method.

In the optimisation of the blood spotting method, experiment was carried out where in 6 mm diameter discs were punched using Sole 6 mm punch [Model PF35A0G1] (Rapesco, Sevenoaks, England) from Guthrie cards (blank and spotted with non-spiked whole blood, $n = 10$) and the weight of each disc was measured using Sartorius Microbalance ME5. The mean weight of the punched DBS discs was calculated followed by measurement of standard deviation and coefficient of variation.

2.4. Chromatographic and mass spectrometric (MS) conditions

Chromatographic separation was achieved using reverse phase chromatography with gradient elution. A Waters Alliance® High Throughput System (Waters Ltd., Watford, UK) with built in Alliance 2795 Separations Module coupled with a Waters Micromass Quattro Premier™ XE tandem quadrupole mass spectrometer (Waters, Manchester, UK) was used for chromatographic separa-

tion and analysis. Two ionisation options i.e. electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) were available on the mass spectrometer. The LC system and the mass spectrometer were both controlled by MassLynx™ 4.0 Software with the QuanLynx Application Manager.

The HPLC column used in the method was a Waters Sunfire C18 reversed-phase column (2.1 mm × 150 mm, particle size 3.5 μm) preceded by a (2.1 mm × 10 mm, particle size 3.5 μm) guard column of matching chemistry. An isocratic elution using mobile phase consisting mixture of methanol and water (60:40, v/v) was pumped at a flow rate of 0.3 ml/min up to 11 min for elution of CAN and MT. At the end of each run, high percentage organic wash (methanol:water 95:5, v/v) was employed for 3 min. This vigorous increase in the organic content was intended to elute late impurities at a faster rate. After organic wash, the composition of the mobile phase was brought back to the original methanol:water (60:40, v/v) and this was held to 20 min so as to re-equilibrate the column.

The column temperature was kept at 35 °C. The sample injection volume employed was 40 μl. The different MS parameters were optimised during flow injection (syringe pump; 10 μl/min) of standard CAN and MT solutions (1 μg/ml) prepared in mobile phase into the LC mobile phase (pumped at 0.3 μl/min). During the tuning, the intensity of the base peak for each compound was monitored and adjusted to maximum.

In the earlier attempts at method development, CAN and MT were ionised using the ESI source in the positive ion mode. This did not give reproducible results and subsequently an APCI probe in the positive ion mode was used to ionise CAN and MT. The following optimised MS operating conditions were used: nebulising gas (nitrogen), 100 l/h; desolvation gas (nitrogen), 400 l/h; source temperature, 120 °C; APCI probe temperature, 650 °C; corona current, 0.1 μA; cone voltage, 35 V for CAN and 30 V for MT. Analysis was carried out by selected-ion monitoring (SIM) mode. The $(M+H)^+$ ions at m/z 341.1 and 303.3 were selected as detection ions for CAN and MT, respectively. The dwell time for each ion was set at 0.1 s.

2.5. Patient sampling

The study protocol was approved by the Ethics Committee of the School of Medicine/Queen's University Belfast. Eligible patients (premature neonates, infants or children up to 12 years of age) were those who had been hospitalised and given intra-venous potassium canrenoate as a part of their clinical management. Informed verbal and written consent was obtained from the parent(s) or guardian(s) of all eligible patients. Assent was obtained from paediatric patients who were 8 years or older. In the case of infants and children, samples were collected from each patient at times when other blood samples were being withdrawn for routine laboratory analysis. However, if routine samples were not required during the course of the treatment, the ethical approval allowed samples to be taken at the discretion of the research nurse. From these paediatric patients up to 1 ml of blood was collected. To prepare a DBS sample, 30 μl of blood were spotted onto a Guthrie card and allowed to dry for 3–4 h in the dark at ambient temperature and then stored at −20 °C until analysis.

In the case of premature neonates in the Neonatal Intensive Care Unit, blood spot samples were collected from heel prick; however, in some cases a small volume of blood was collected from an indwelling venous or arterial line. The DBS sample was prepared as detailed above.

2.6. Sample preparation

A 6 mm disc was punched from the DBS, ensuring that an area completely filled with blood was obtained. The disc was transferred to a 5 ml disposable borosilicate glass culture tube to which 2 ml of methanolic IS solution (7.5 ng/ml) was added. The sample was extracted for 60 min with intermittent shaking every 20 min. After the extraction period, the disc was removed and the extracted solution evaporated to dryness under the gentle stream of nitrogen at 37 °C using a Zymark TurboVap® LV Concentration Workstation (Hopkinton, MA). The dry residue thus obtained was reconstituted in 1 ml of acetonitrile/water (at ratio 1:9, v/v) mixture.

The reconstituted sample was further subjected to solid phase extraction (SPE) using Oasis® HLB cartridges (1 cm^3/30 mg) on a Waters Extraction Manifold (Waters, USA). The HLB cartridges were conditioned by passing 1 ml of methanol, followed by equilibration with 1 ml of water. The reconstituted sample (1 ml) was loaded on to the cartridges. The loaded samples were washed in two steps firstly, 1 ml of 5% methanol was used followed by 1 ml of 60% methanol. Finally, 1 ml of methanol was used for the elution step. The eluate thus obtained was again evaporated to dryness under the gentle stream of nitrogen at 37 °C using a Zymark TurboVap® LV Concentration Workstation. The dry residue thus obtained was reconstituted in 100 μl of mobile phase and 40 μl was injected for LC–MS analysis.

2.7. Validation procedures

All validation experiments were performed according to the ICH guideline Q2B [34].

2.7.1. Selectivity

The selectivity of the developed method was tested by analysing six batches of blank DBS samples. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectroscopic conditions and compared with those obtained with an aqueous solution of the analyte at a concentration near the lower limit of quantification (LLOQ). This was to ensure that no interfering peaks were present in the biological matrix at the retention times of CAN and MT.

2.7.2. Linearity

The linearity of the developed method was evaluated by analysing CS samples of CAN on three separate days. Each calibration curve consisted of a blank sample (blank DBS sample processed without internal standard), a zero sample (blank DBS processed with internal standard) and was generated from eight non-zero samples covering the expected range (25–1000 ng/ml). Plots of peak area ratio (response) against the respective analyte concentration were used to assess the relationship between the response and concentration. Calibration curves (area ratio with the internal standard versus nominal concentration) were fitted by least-squares linear regression using 1/concentration^2 ($1/X^2$) as the weighting factor to calculate the slope, intercept and correlation coefficient (r).

2.7.3. Accuracy and precision

The accuracy and precision of the developed method was determined by replicate analysis ($n = 3$) of QC DBS samples spiked with CAN at four concentrations i.e. LLQC (25 ng/ml), LQC (50 ng/ml), MQC (250 ng/ml) and HQC (1000 ng/ml) on three different days. Within-day accuracy and precision were calculated on a single day using three replicates at each concentration level. Between-day accuracy and precision were calculated using three replicates at each concentration level over three consecutive days. The accuracy of the method was calculated by comparing the measured concentrations with the nominal (true) concentration and was expressed as the mean percent relative error (RE %). The precision of the method was expressed as the percent coefficient of variation (CV %).

2.7.4. Sensitivity (limit of detection (LOD) and limit of quantification (LOQ))

LOD and LOQ values were calculated based on the standard deviation of the response (σ) and the slope (S) of calibration curves prepared for the CAN at levels approximating the limit of detection (10–100 ng/ml) according to the following equations:

$$\text{LOD} = 3.3\left(\frac{\sigma}{S}\right)$$

$$\text{LOQ} = 10\left(\frac{\sigma}{S}\right)$$

where σ was estimated from the standard deviation of the y-intercepts of the regression lines. For practical purposes, LLQC (25 ng/ml) was assessed for acceptable accuracy (<20% RE) and precision (<20% CV), designated as LLOQ.

2.7.5. Matrix effect, recovery and process efficacy

The influence of matrix on APCI and the quantification of CAN and MT was monitored using both qualitative and quantitative methods. The post-column infusion method was used as the qualitative method. For this purpose, a solution of CAN/MT (100 ng/ml, each) was continuously infused post-column using a syringe pump at a flow of 10 μl/min and mixed with the column effluent through a T joint before entering the ionisation interface. Blank DBS samples were processed according to the sample preparation procedure and 40 μl were injected under the described chromatographic and spectroscopic conditions to analyse a potential influence of eluting matrix components onto analyte responses.

The quantitative method [35] involved a comparison of:

(a) The instrument response for the calibrators (including the IS) injected directly in mobile phase (neat solutions).
(b) The same amount of compound added to extracted blank samples (post-extraction spiked samples).
(c) The same amount of analyte added to the biological matrix before extraction (pre-extraction spiked samples).

The absolute matrix effect was measured by comparing the responses from post-extraction spiked samples with those from pure solutions at three pre-determined concentrations of 50, 100 and 500 ng/ml of CAN. If the ratio is <85% or >115%, an exogenous matrix effect is implied [36].

$$\%\ \text{Matrix Effect} = \frac{\text{Response}_{\text{post-extraction spiked sample}}}{\text{Response}_{\text{neat solution}}} \times 100$$

Recovery was determined at three pre-determined concentrations of 50, 100 and 500 ng/ml of CAN by comparing the MS response of the pre-extraction spiked samples with those spiked post-extraction into a blank matrix. Because both samples have the matrix ingredients present, the matrix effect can be considered the same for pre-extraction spiked samples and post-extraction spiked samples and any difference in responses is caused by extraction recovery.

$$\%\ \text{Recovery} = \frac{\text{Response}_{\text{pre-extraction spiked sample}}}{\text{Response}_{\text{post-extraction spiked sample}}} \times 100$$

Overall process efficiency was measured at three pre-determined concentrations of 50, 100 and 500 ng/ml of CAN by comparing the responses from pre-extraction spiked samples with those from neat solutions. This term accounts for any loss in signal attributable to the extraction process or matrix effect.

$$\%\ \text{Process Efficiency} = \frac{\text{Response}_{\text{pre-extraction spiked sample}}}{\text{Response}_{\text{neat solution}}} \times 100$$

2.7.6. Stability

Stock solution stability was tested at the selected storage condition (−20 °C) after a period of one month. The stock solution was considered stable if 95–105% of the nominal concentration was found when compared with a freshly prepared stock solution. Stability testing of spiked DBS samples at −20 °C was carried out over a period of one month at all four QC levels. Bench top stability of processed QC samples was carried out at all four QC levels up to 12 h.

2.8. Clinical application

The developed and validated method was applied to the analysis of CAN in neonatal and paediatric DBS samples (n = 160). The samples, stored at −20 °C were allowed to reach room temperature before being extracted.

3. Results and discussion

3.1. Method development

In the early stage of the studies, an ESI probe in positive mode was used for the development of the assay. CAN was tuned to obtain various MS conditions. In the full scan mode, the protonated molecular ion $(M+H)^+$ of CAN was observed at a m/z ratio of 341.1 along with other adducts. The fragmentation of the protonated ion was carried out using Argon as the collision gas. A fragment ion having a m/z ratio of 106.9 was selected as it gave higher intensity during the optimisation of MS parameters. Thus 341.1 > 106.9 transition was used to monitor the CAN in the method. However, it was found that sensitivity was insufficient and dropped during repeated injections. Results were not reproducible and calibration curves were non-linear over the expected calibration range of 25–1000 ng/ml. This observation was in agreement with previously published results by other researchers [25].

MT was used as the IS in this work since, at the commencement of this study, no labelled analogues of CAN were available (as far as authors were aware). During the final preparation of this manuscript we were made aware of the availability of such analogues which, had they been used, may well have reduced the overall run time of this analysis.

To overcome the problems associated with the ESI technique, the APCI technique in positive ion mode was investigated for estimation of CAN. In the full MS scan the molecular ions of CAN and MT at m/z ratios of 341.1 and 303.3, respectively, were the dominant ions. These base peaks were of higher intensity and stability compared to those obtained with the ESI technique. Furthermore, the SIM (MS) acquisition mode resulted in larger peak areas and better sensitivities for both CAN and MT compared to the MRM (MS/MS) acquisition mode. This observation can be related to the low abundance of fragment ions which were observed during the optimisation of the MRM conditions. Therefore, the LC–MS method was chosen for the quantification and taken further for method validation.

For optimisation of the chromatographic separation, attempts were made to use short and narrow HPLC columns including Xterra® MSC18 (50 mm × 2.1 mm, 2.5 μm) and Xbridge® C18 (50 mm × 2.1 mm, 2.5 μm) with isocratic and gradient elution in an attempt to achieve efficient run times. However, optimum separation was observed with the longer Sunfire C18 column (150 mm × 2.1 mm, 3.5 μm) which was chosen to ensure better resolution between CAN, MT and endogenous compounds.

During the method development with DBS samples, an isocratic method was developed with a total run time of 10 min. However, when extracted blank DBS samples were monitored (at m/z 341.1)

for longer periods of time (up to 60 min) to check for the presence of any latent peaks, a peak was observed at 35 min. The origin of this peak was found to be related to a component extracted from the Guthrie cards used for the collection of the blood spots. Thus, to avoid any interference in the subsequent injections, a high organic wash (as detailed in Section 2) was employed at the end of each sample in order to reduce the elution time of this latent peak. This approach also helped in cleaning the column from any accumulating phospholipids or lipoproteins which are known to shorten the column life and cause matrix effects [36]. The column was re-equilibrated to original mobile phase composition before being continued to subsequent sample injections. The retention time of CAN and MT were found to be 7.5 and 10.3 min respectively with a total run time of 20 min.

3.2. Blood spotting procedure

The blood spotting procedure was found to be satisfactory. Investigation of drying conditions for the blood spots applied to the Guthrie cards indicated that drying for 3 h in the dark resulted in extracts which gave the lowest % CV. Investigations of the weights of cut discs ($n = 10$) from the Guthrie cards, both blank and spotted with non-spiked whole blood, showed excellent reproducibility—blank 5.17 ± 0.17 mg (CV; 2.49%); non-spiked whole blood 7.78 ± 0.34 mg (CV; 4.43%). The low % CV indicated the precision of the disc cutting procedure. In a previous study we have shown that the volume (20–100 μl) of drug-spiked blood applied to a Guthrie card does not result in any significant variation in estimated concentration when the same disc size is used for determination the drug concentration.

3.3. Sample preparation

Sample clean-up was found to be the most important step in achieving higher sensitivity with the method as well as reducing interference in actual clinical sample analysis. Unlike any other liquid biomatrix, the DBS technique poses significant challenges for the extraction of analytes. It is important that the analyte is partitioned into the extraction solvent. However, along with analyte, phospholipids and various other endogenous impurities from whole blood are also partitioned into the extraction solvent. The solvent extraction process also results in extraction of significant impurities from the paper discs.

In the initial sample preparation work, sample extraction from DBS using various solvents and their mixtures was investigated. While developing the sample clean-up method, matrix-effect studies were also conducted simultaneously to check the noise/background and its effect on sensitivity of the method. While the extraction methods resulted in high percentage recoveries from DBS some significant interferences were observed and thus further clean-up was required.

SPE offers the most suitable approach to sample clean-up of this matrix. Evaporation and reconstitution steps were thus introduced after initial solvent extraction of DBS disc with methanol. The residue from the initial extraction was reconstituted in aqueous–organic solvent mixture (acetonitrile–water 1:9, v/v) and subjected to SPE analysis. Various SPE cartridges such as HLB and MCX were tested so as to obtain optimum recovery and minimum impurities in the final reconstituted sample. HLB cartridges were investigated taking into consideration the lipophilicity of CAN and MT, while MCX cartridges were investigated as they reportedly minimise the matrix effects.

In order to compare the different SPE methods, the matrix effect was assessed by using a surrogate method for monitoring the elution profiles of the phospholipids [36,37]. For this purpose, two major phospholipids, glycophosphocholines and 2-lysoglycophosphocholines at m/z ratio of 184 and 104 were added to the SIM mode of analysis along with CAN and MT and monitored [36]. Based on these experiments, it was found that the use of MCX cartridges did not offer any advantage over the HLB cartridges. This could be related to the neutral nature of the analytes. Thus finally HLB cartridges were selected and further used in validation of the method.

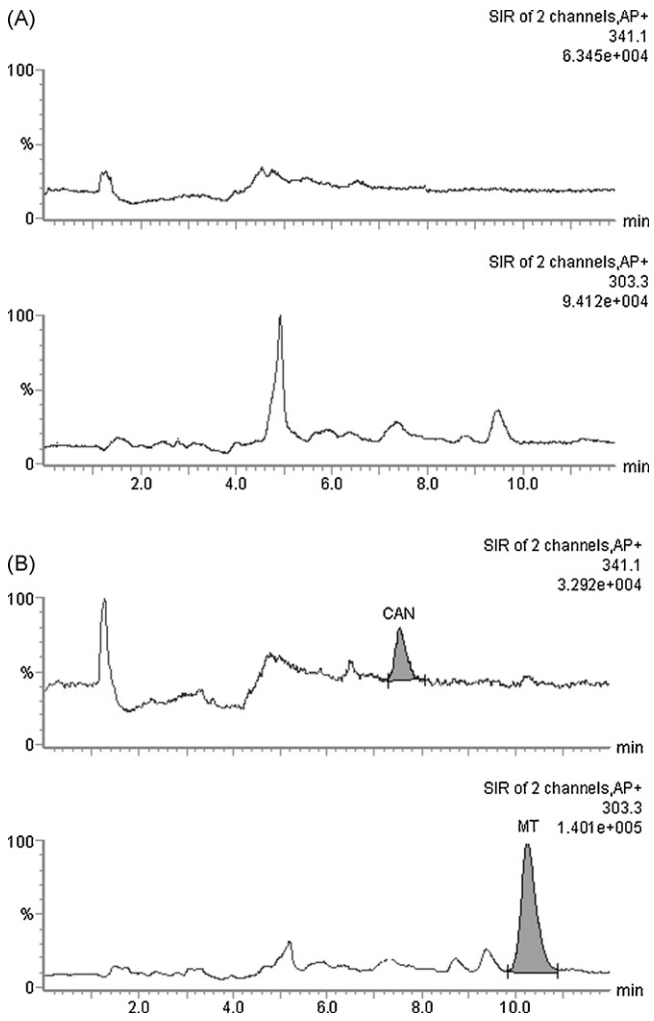

Fig. 2. Representative SIM chromatograms of a blank DBS (A), DBS spiked with CAN at LLOQ (25 ng/ml) and MT (B) [m/z 341.1: CAN; m/z 303.3: MT].

3.4. Method validation

3.4.1. Selectivity

The chromatograms for blank DBS, blank DBS spiked with CAN at LLOQ (25 ng/ml) and MT (IS) are shown in Fig. 2. The chromatogram of a patient DBS sample is shown in Fig. 3. It can be seen from the figures that, no interfering peaks were observed at the retention times of CAN and MT. The extraction method showed very good selectivity for the analysis of CAN and MT. Patient samples also showed appropriate separation of CAN and MT and were devoid of any interference peaks.

3.4.2. Linearity

The calibration curve of CAN was found to be linear over the selected concentration range of 25–1000 ng/ml with a mean r >0.994 ($n = 3$). The mean slope and intercept values from the three calibration curves were 0.120 and 7.198, respectively.

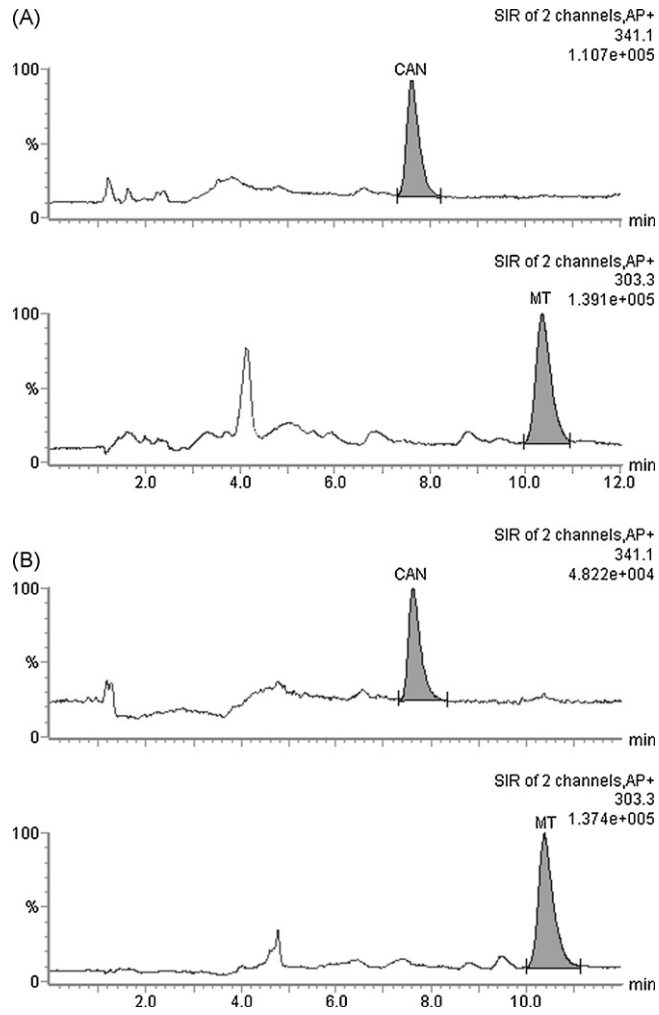

Fig. 3. SIM chromatograms of two patient DBS samples taken at 2.08 h (A, found concentration 663.5 ng/ml) and 0.5 h (B, found concentration 233.7 ng/ml) after IV administration of potassium canrenoate.

3.4.3. Accuracy and precision

Data on within day and between-day accuracy and precision of the method for determination of CAN are presented in Table 1. Within-day accuracy (%RE) over four QC concentrations ranged from −2.95 to 7.31, while between-day accuracy (%RE) ranged from 0.03 to 7.52. Within-day precision (% CV) ranged from 0.18 to 5.73, while between-day precision (% CV) ranged from 3.46 to 7.78. Overall, accuracy and precision values for within and between-day were <20% at LLQC and <15% at all other concentrations tested. Thus it can be claimed that, the method possessed good accuracy and precision.

3.4.4. Sensitivity (limit of detection and limit of quantification)

Calculated values for LOD and LOQ from calibration curves prepared for the CAN at levels approximating the limit of detection (10–100 ng/ml) were found to be 6.1 ± 1.4 and 18.5 ± 3.1 ng/ml respectively. However, for practical purposes a LLQC (25 ng/ml) was validated as the LLOQ. The accuracy (% RE) and precision (% CV) at LLQC were found to be −2.95 and 5.73 respectively. The observed values were less than the specified limit of <20%, thus 25 ng/ml was considered as the validated LOQ of the method.

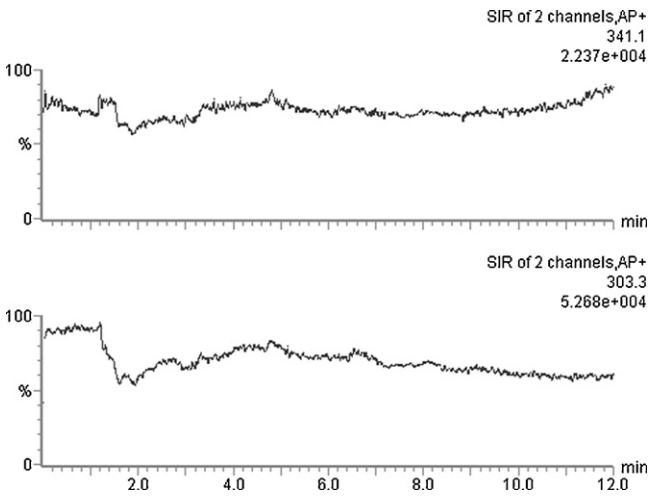

Fig. 4. Post-column infusion assessment of matrix effect.

3.4.5. Matrix effect, recovery and process efficacy

The matrix effect is defined as the analyte ionisation suppression or enhancement due to the presence of the matrix components that could originate from the endogenous compounds, metabolites, co-administered drugs, dosing vehicles, mobile phase additives and plastic tubes [36]. Matrix effects can be experienced with both ESI and APCI, however, they are more pronounced with ESI [38]. The assessment of the matrix effect constitutes an important and integral part of validation for a quantitative LC/MS method for supporting pharmacokinetic studies [35,39] and the FDA Guidance for Industry on Bioanalytical Method Validation requires the assessment of matrix effect during method validation for quantitative LC/MS or LC/MSMS methods [40].

Results of the qualitative method are shown in Fig. 4, which represents the SIM traces obtained during the post-column infusion experiment. The results indicate that there were no significant baseline variations at the retention times of both CAN and MT. In addition, based on the quantitative method, the estimated matrix effect (% ME) on both CAN and MT was found to be within 85% and 115% (Column 2, Table 2), which indicates the lack of any major ion suppression on enhancement for CAN and MT in this method.

Table 2 also shows that the overall mean recovery for CAN from DBS at pre-determined concentrations of 50, 100 and 500 ng/ml. It can be seen from the table that all the recovery values at the three concentration levels were consistent and reproducible. The values were found to be approaching 100% and variability (% CV) at each concentration was found to be less than 10%. The recovery of the IS

Table 1
Results of within and between-day accuracy and precision

Nominal concentration (ng/ml)	Within day			Between day		
	Measured concentration (ng/ml)	Accuracy % RE	Precision % CV	Measured concentration (ng/ml)	Accuracy % RE	Precision % CV
25 (LLQC)	24.26 ± 1.39	−2.95	5.73	25.01 ± 1.62	0.03	6.49
50 (LQC)	53.63 ± 0.43	7.31	0.80	52.41 ± 4.08	4.81	7.78
250 (MQC)	232.99 ± 0.42	−6.80	0.18	233.66 ± 9.57	7.52	4.10
1000 (HQC)	1027 ± 14.11	2.77	1.37	1008.4 ± 34.89	0.84	3.46

Values are given as mean ± SD of three determinations ($n = 3$)

Table 2
Matrix effect (ME), recovery (REC) and process efficiency (PE) of CAN and MT

Spiked concentration (ng/ml)	% ME ($n = 3$)		% REC ($n = 3$)		% PE ($n = 3$)	
	Mean ± SD	% CV	Mean ± SD	% CV	Mean ± SD	% CV
50 (CAN)	103.0 ± 0.3	0.3	114.2 ± 2.1	1.9	117.6 ± 2.5	2.1
100 (CAN)	98.6 ± 1.6	1.7	101.2 ± 7.0	6.9	99.9 ± 8.6	8.6
500 (CAN)	109.4 ± 1.2	1.1	103.6 ± 0.4	0.4	113.3 ± 1.6	1.4
100 (MT)	90.6 ± 7.2	7.9	82.9 ± 5.3	6.4	75.2 ± 8.3	11.1

was found to be 83%. The method displayed good overall process efficiency of more than 100% for CAN. Thus these findings support the use of this extraction procedure for paediatric DBS samples.

3.4.6. Stability

Stock solution stability of CAN and MT showed no change in assay value over a period of one month, indicating that both the drugs were stable at 1 mg/ml concentration at −20 °C. Assay of stability samples of spiked DBS samples at LLQC, LQC, MQC and HQC ($n = 3$) at one month showed values comparable with freshly prepared DBS samples. Both accuracy and precision values at all QC levels were found be within the limit (<20% for LLQC and <15% at all other QC concentrations). Bench top stability of processed samples at all four QC levels showed <1% variation when responses of CAN were compared at 0 time and at 12 h, indicating no degradation of the CAN and MT over the specified period in reconstituted mobile phase.

3.4.7. Clinical application

The selective and sensitive method thus developed and validated was clinically validated by application of the method to actual neonatal and paediatric DBS samples. A total of 160 DBS samples collected from 37 neonatal and paediatric patients were analysed using the method described. No major difficulties were identified during analysis of the clinical DBS samples. The concentration range for all 160 samples analysed by the proposed method was found to be 34–663.5 ng/ml. The lowest patient sample concentration was well above the LLOQ (25 ng/ml) of the method. None of the patient samples were above the highest calibration point (1000 ng/ml) of the method. Fig. 3 shows SIM chromatograms of two patient DBS samples taken at 2.08 h (A) and 0.5 h (B) after IV administration of potassium canrenoate. The concentrations found for these two samples are 663.5 and 233.7 ng/ml respectively. The concentration data for all DBS samples measured during the study was further subjected to Population PK study by using nonlinear mixed effect modelling (computer program NONMEM®, version VI, level 1.0). This PK study will be the subject of a further publication.

4. Conclusion

This is a first report describing the quantitative estimation of CAN in very low volume neonatal and paediatric clinical samples. The novel bioanalytical method, employing DBS as the sample collection medium with LC–MS detection led to a selective and sensitive method appropriate for use in paediatric and neonatal samples. The selection of the correct ionisation technique (APCI vs ESI) played an important role in the successful method development and validation. The method was shown to be linear, accurate, precise and robust. The method was found to be selective and sensitive enough (validated LOQ 25 ng/ml) to extract and quantify CAN in 6 mm DBS discs containing approximately 10 μl of whole blood. The sample processing technique developed was effective in the extraction of CAN and MT with minimal matrix effects. Quantitative and qualitative estimation of matrix effects further proved the selectivity of the method. The method is suitable for pharmacokinetic and other clinical research studies involving CAN.

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