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Gas chromatography/negative ion chemical ionization mass spectrometry and liquid chromatography/electrospray ionization tandem mass spectrometry quantitative profiling of *N*-acetylcysteine conjugates of valproic acid in urine: application in drug metabolism studies in humans†

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We report a GC/NICI-MS assay and a LC/ESI-MS/MS assay for the analysis of *N*-acetylcysteine (NAC) conjugates of (*E*)-2,4-diene VPA (NAC I and NAC II) identified in humans. The assay also includes the analysis of the NAC conjugate of 4,5-epoxy VPA (NAC III), an identified metabolite in rats treated with 4-ene VPA for its use in metabolic studies in animals. The highly sensitive GC/MS assay was designed to monitor selectively the diagnostic and most abundant $[M - 181]^-$ fragment anion of the di-PFB derivatives of NAC I, NAC II, and NAC IV, the internal standard (IS) and the PFB derivative of NAC III. The higher selectivity of LC/MS/MS methodology was the basis for an assay which could identify and quantitate the underivatized conjugates simultaneously using MRM of the diagnostic ions *m/z* 130 and 123 arising from the CID of their protonated molecular ions $[MH]^+$. The GC/MS assay employed liquid–liquid extraction whereas the LC/MS/MS assay used a solid-phase extraction procedure. Linearity ranges of the calibration curves were 0.10–5.0 μg ml^{-1} by GC/MS and 0.10–1.0 μg ml^{-1} by LC/MS/MS for NAC I, NAC II and NAC III (r^2 = 0.999 or better). Both assays were validated for NAC I and NAC II and provided good inter- and intra-assay precision and accuracy for NAC I and NAC II. The LOQ by LC/MS/MS was 0.1 μg ml^{-1}, representing 1 ng of NAC I and NAC II. The same LOQ (0.1 μg ml^{-1}) was observed by GC/MS and was equivalent to 100 pg of each metabolite. NAC III was detected at concentrations as low as 0.01 μg ml^{-1} by both methods. The total urinary excretion of the NAC conjugates in four patients on VPA therapy was determined to be 0.004–0.088% of a VPA dose by GC/MS and 0.004–0.109% of a VPA dose by LC/MS/MS.

KEYWORDS: valproic acid, *N*-acetylcysteine conjugates; gas chromatography; liquid chromatography/tandem mass spectrometry; positive electrospray ionization; negative ion chemical ionization

INTRODUCTION

Valproic acid (VPA) is a broad-spectrum antiepileptic agent and its use is limited by reported incidences of hepatotoxicity.[1] The side effect is believed to be mediated through the bioactivation of the metabolites, 4-ene VPA and (*E*)-2,4-diene VPA, with the latter appearing as *N*-acetylcysteine (NAC) conjugates in urine.[2,3] The NAC conjugates of (*E*)-2,4-diene VPA, i.e. 5-NAC-3-ene VPA (NAC I) and 5-NAC-2-ene VPA (NAC II), have been identified in urine samples from patients on VPA[3] (S. V. Gopaul *et al*., *Drug. Metab. Dispos.*, in press). Interestingly, 5-NAC-4-OH-VPA-γ-lactone (NAC III), a

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† Abbreviations: VPA, valproic acid (2-propylpentanoic acid); 4-ene VPA, 2-propyl-4-pentenoic acid; 2-ene VPA, 2-propyl-2-pentenoic acid; 3-ene VPA, 2-propyl-3-pentenoic acid; (*E*)-2,4-diene VPA, (*E*)-2-propyl-2,4-pentadienoic acid; FVPA-GLN, 2-fluoro-2-propylpentanoylglutamine; 4,5-epoxy VPA, 2-propyl-4,5-epoxypentanoic acid; 4,5-diOH-VPA-γ-lactone, 3-propyl-5-hydroxymethyltetrahydro-2-furanone; GSH, glutathione; IS, internal standard; NAC, *N*-acetylcysteine; NAC I, (*E*)-5-*N*-acetylcystein-*S*-yl-3-ene-VPA; NAC II, (*E*)-5-*N*-acetylcystein-*S*-yl-2-ene-VPA; NAC III, 4-hydroxy-5-*N*-acetylcystein-*S*-yl-VPA; NAC IV, 1-*N*-acetylcystein-*S*-ylacrylic acid; PFBBr, pentafluorobenzyl bromide; PFB, pentafluorobenzyl; PPA, propionic acid; TFA, trifluoroacetic acid; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC, liquid chromatography; GC, gas chromatography; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; NICI, negative ion chemical ionization; SIM, selected ion monitoring; CID, collision-induced dissociation; LOQ, limit of quantification; SPE, solid-phase extraction.

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prominent metabolite of 4-ene VPA in rats, [4] was absent in patients on VPA (S. V. Gopaul *et al*., *Drug. Metab. Dispos.*, in press).

Because NAC conjugates in urine are frequent biochemical end products of the GSH conjugates of reactive metabolites, they are expected to be found in elevated amounts in patients suffering from VPA-induced hepatotoxicity or those at higher risk of the disease. Thus, in order to investigate this theory, there is a vital need to develop robust, reproducible, and reliable assays to measure all three conjugates both in humans and animals.

Figure 1 shows the structures of NAC I, NAC II and NAC III as determined on the basis of detailed chromatographic and spectroscopic evidence[3–5] (S. V. Gopaul *et al*., *Drug. Metab. Dispos.*, in press). In each case, the terminal carbon of the short chain fatty acid structure is conjugated to the sulfur atom of the NAC moiety. Whereas NAC I and NAC II are unsaturated dicarboxylic acids, NAC III is a carboxy lactone with one free carboxylic acid group. The structures of the IS, NAC IV and FVPA-GLN are also shown.

In general, the quantitation of VPA and its metabolites is based on methods designed for long-chain fatty acids and prostaglandins.[6–11] These methods rely on the enhanced sensitivity of the $[M - PFB]^-$ carboxylate anion formed from the negative ion chemical ionization (NICI) of the PFB derivatives of the acids. Thus, based on the same principle, the quantitation of NAC I, NAC II and NAC III can be achieved simultaneously using selected ion monitoring (SIM) of the $[M - 181]^-$ carboxylate anion of their PFB esters.

The characterization of thiol conjugates of xenobiotics has been carried out largely by LC/MS/MS.[5,12–17] Under positive electrospray ionization (ESI), protonated molecular ions of NAC conjugates give rise to product ion spectra dominated by the cleavage of a thioether bond characterized by *m*/*z* 130 and corresponding to the loss of H_2S from MH^+ ion of NAC[5] (S. V. Gopaul *et al*., *Drug. Metab. Dispos.*, in press). The cleavage of the second carbon–sulfur bond produces the product ion at *m*/*z* 123 for NAC I and NAC II. Therefore, quantitative analysis of the conjugates can be carried out in a single run by monitoring these diagnostic ion transitions.

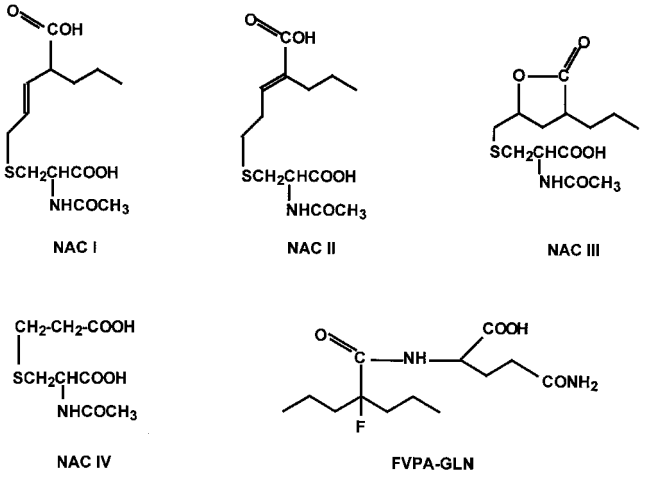

Figure 1. Structures of NAC I, NAC II, NAC III, NAC IV and FVPA-GLN.

We describe here two assays for the measurement of NAC conjugates of (*E*)-2,4-diene VPA, a reactive metabolite of VPA, in human urine using GC/MS and LC/MS/MS. These assays are important fundamentally to the study of VPA hepatotoxicity in humans and animals.

EXPERIMENTAL

Materials

Chemicals synthesized in the laboratory. NAC I, NAC II and NAC III were synthesized according to procedures described previously[3,5] (S. V. Gopaul *et al*., *Drug. Metab. Dispos.*, in press). The IS for the LC/MS/MS assay was FVPA-GLN, which was synthesized in our laboratory.[18]

The methyl NAC conjugate of methyl acrylate was synthesized in this laboratory.[3] The IS for the GC/MS assay, NAC IV, was obtained by hydrolyzing an aliquot of the dimethyl ester of the NAC conjugate of acrylic acid with two aliquots of sodium hydroxide at room temperature for 24 h and the solution was made up to volume for the required concentration with distilled water. The identity of the free acid was confirmed by GC/NICI-MS prior to analysis.

Chemicals purchased. Pentafluorobenzyl bromide (PFBBr) and diisopropylethylamine (DIPEA) were purchased from Aldrich Chemical (Milwaukee, WI, USA). Trifluoroacetic acid (TFA) and propionic acid (PPA) were purchased from Sigma Chemical (St Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Instrumentation

GC/NICI-MS analysis was performed on a Hewlett-Packard (HP) 5989 mass-selective detector coupled to a Model 5890 Series II gas chromatograph. Analysis was carried out on a J & W Scientific (Folsom, CA, USA) DB-1701column (30 m × 0.32 mm i.d. with film thickness 0.25 μm). The oven temperature program was as follows: 50–150 °C at 20 °C min^{-1}, held at 150 °C for 5 min, 150–300 °C at 10 °C min^{-1}, held at 300 °C for 5 min. The injection port temperature was 240 °C, the source temperature 200 °C and the interface temperature 280 °C. The pressure of the carrier gas, helium, was set at 10 psi.

For NICI analysis, the ion source temperature was kept at 200 °C and the interface temperature was set at 280 °C. The reagent gas, methane, was maintained at 1 Torr (1 Torr = 133.3 Pa). The emission current was 300 μA and the ionization energy was 120 eV.

LC/MS/MS experiments were carried out on a Micromass (Montreal, Quebec, Canada) Quattro triple-quadrupole mass spectrometer interfaced to an HP 1090 Series II HPLC system equipped with a Phenomenex (Torrance, CA, USA) C_8 column (100 mm × 2.1 mm i.d., 5 μm). The HPLC conditions employed mobile phase A, methanol–water (43 : 57) with 0.025% each of TFA and PPA. Mobile phase B consisted of methanol. At time zero, mobile phase A was pumped isocratically for 30 min. Mobile phase B was increased from 0 to 100% at 30.1 min

and held for 5 min, followed by a sharp gradient increase of mobile phase A to 100% at 35.1 min. All flow-rates were 0.1 ml min^{-1}.

The MS/MS conditions employed positive electrospray as the ionization energy. The HPLC eluate was introduced into the stainless-steel electrospray capillary sprayer held at 3.5 kV. The mass spectrometer was operated for the purpose of MRM with multipliers 1 and 2 set at 650 V, cone voltage at 22 kV and skimmer offset by 5 V. The low- and high-mass resolution were set at 5 during quantitative analysis.

Assay for the quantitative profiling of thiol conjugates in the urine samples of patients on VPA therapy

Working solutions. Two working solutions of NAC I, NAC II and NAC III were prepared at concentrations of 0.1 and 0.01 mg ml^{-1}. Working solutions of NAC IV and FVPA-GLN at 0.1 mg ml^{-1} were also prepared individually. The derivatizing reagent (PFBBr) was prepared by dissolving 400 μl of the neat reagent in 1 ml of anhydrous ethyl acetate. While the assay was for the simultaneous analysis of NAC I, NAC II and NAC III, it was validated for NAC I and NAC II, the only two conjugates of VPA found in humans. The assay of NAC III could be used for animal studies where the conjugate is a detectable metabolite.

Preparation of calibration curves. The calibration curves for the GC/MS assay were prepared by adding an appropriate amount of the working solutions to test-tubes to yield standard concentrations of 0.10, 0.25, 0.50, 0.75, 1.0, 2.5 and 5.0 μg ml^{-1} of each conjugate in 1 ml of control urine sample. Each tube was spiked with 20 μl of 0.1 mg ml^{-1} NAC IV. The analysis was conducted by SIM of the $[M-181]^-$ fragment of each PFB-derivatized conjugate, i.e. *m/z* 482 for NAC I and NAC II, *m/z* 302 for NAC III and *m/z* 414 for NAC IV. The calibration curves were constructed by plotting the area ratios of each analyte to the IS against the respective concentration of the conjugate.

The calibration curves for the LC/MS/MS assay were prepared by adding an appropriate amount of the working solutions to test-tubes to yield standard concentrations of 0.10, 0.25, 0.50, 0.75 and 1.0 μg ml^{-1} of each conjugate in 1 ml of control urine sample. Each tube was spiked with 10 μl of 0.1 mg ml^{-1} FVPA-GLN. LC/MS/MS calibration curves for the NAC conjugates were constructed by plotting area ratio of the conjugates to the IS at product ion *m/z* 130 arising from the CID of their respective $[MH]^+$. The calibration curves were also evaluated using product ion *m/z* 123 for NAC I and NAC II. The areas of both isomers of NAC I were added and plotted as total NAC I. All calibration curves were assessed by weighted linear regression ($1/y^2$).

Liquid–liquid extraction procedure (GC/MS assay). A 1 ml volume of each calibration standard, blank urine sample and patient sample was acidified to pH 3–4 with concentrated H_3PO_4, saturated with NH_4Cl and extracted with 5 ml of ethyl acetate twice. The organic layers were separated, combined and reduced in volume to 1 ml under nitrogen. The conjugates were derivatized with 10 μl of 40% PFBBr solution and 10 μl of DIPEA in a conical vial at 50 °C for 1 h.

Solid-phase extraction (SPE) extraction procedure (LC/MS/MS assay). The pH of each sample was adjusted to 3–4 with 3 M HCl and allowed to equilibrate for 5–10 min at room temperature. The samples were then applied to C_2 cartridges (500 mg) preconditioned with methanol and water. The cartridges were washed successively with water (pH 3–4) and water–methanol (95 : 5, v/v) to remove endogenous substances which interfered with the chromatography of the conjugates. The latter were then eluted with 3 ml of methanol twice. The combined organic layers were dried under a gentle stream of N_2. The extract was reconstituted with 1 ml of mobile phase.

Validation studies of GC/MS and LC/MS/MS assays

Precision of the assays. The inter-assay precision was determined from the variations observed for (1) the slopes of calibration curves run on six or seven consecutive analytical days and (2) the means of three replicates of three samples each spiked with NAC I and NAC II at concentrations equal to 0.12, 0.42, and 0.80 μg ml^{-1}, respectively. The precision was expressed as the coefficient of variation (CV).

The intra-assay precision was determined from the variations observed for triplicate analyses of two samples at concentrations of 0.12 and 0.80 μg ml^{-1}, respectively, and was expressed as CV.

Accuracy of the assays. The accuracy of the assay was assessed as the percentage of expected values observed for the spiked samples described above.

Recovery of the NAC conjugates prepared for GC/MS assay. Three replicates of two samples were spiked with NAC I to yield concentrations of 1.50 and 3.50 μg ml^{-1} and with NAC II to yield concentrations of 0.44 and 4.50 μg ml^{-1}. A 1 ml volume of each sample was spiked with 20 μl of IS, extracted, derivatized and analyzed as described for the patients' samples. The resulting calculated concentrations were expressed as a percentage of those obtained for the unextracted but identical amounts of NAC I and NAC II spiked in control urine extract, dissolved in ethyl acetate and derivatized.

Recovery of the NAC conjugates prepared for LC/MS/MS assay. Recovery of the conjugates was determined by analyzing triplicates of two samples spiked with NAC I and NAC II at concentrations of 0.25 and 0.80 μg ml^{-1}. The concentrations observed were then expressed as a percentage of those obtained when identical amounts of the conjugates were dissolved in water and analyzed without SPE extraction.

Human study

Four patients, who were treated for epilepsy at British Columbia's Children's Hospital Seizure Clinic in Vancouver, participated in this study. The ages of the patients were 5.3, 5.4, 7.8 and 14.7 years. Patients were on VPA alone or in combination with non-enzyme inducing drugs. Three of the patients were on two divided daily doses of VPA and provided two total urine samples each collected over a 12 h interval. The morning and evening urine collections started immediately after one dose of

VPA and continued until the next dose of the drug was administered. One patient who was on one VPA dose daily provided a 24 h collection. A 1 ml volume of each 12 h urine collection and the 24 h urine collection were analyzed individually and total recovery of NAC conjugates for each patient was calculated and expressed as a percentage of the VPA dose for a 24 h period.

The amount of NAC conjugates recovered by each method was calculated based on the volume of urine collected. The amount of VPA dose (%) recovered as NAC I and NAC II was calculated as described by the equation

$$\text{recovery (\%)} = \frac{\text{amount of NAC conjugates (mol)} \times 100}{\text{amount of VPA per dose (mol)}} \quad (1)$$

RESULTS AND DISCUSSION

The LC/MS/MS assay, developed for the quantitation of NAC I, NAC II and NAC III, is a combination of an efficient SPE extraction procedure and sensitive MS/MS detection using ESI. The assay allowed the simultaneous identification and quantitation of the conjugates with a high degree of selectivity.

The SPE extraction procedure was fast and convenient, requiring very few steps, and is based on the extraction procedure described for the isolation of F_2-isoprostanes.[19] HCl was used to adjust the pH of the urine matrix to 3–4 to optimize the extraction efficiency and to minimize the hydrolysis of the conjugates including the IS, FVPA-GLN. The conjugates were in an acidic medium for a short time (10 min) prior to extraction. The LC/MS/MS response obtained for the conjugates following extraction remained stable and consistent throughout the study.

The urine samples of the patients on VPA and in combination with other drugs appeared to contain lipid-type substances which initially interfered significantly with the assay. A combination of C_2 SPE cartridges, a water–methanol (95 : 5) wash and utilization of a C_8 HPLC column proved to be highly efficient in cleaning up the samples, stabilizing retention time shifts and producing a high recovery (~100%) of the conjugates and IS. A combination of TFA and PPA (0.025%) was necessary to maintain the mobile phase at pH 3 and to increase the sensitivity of NAC I while facilitating baseline separation of its isomers.

Generally, thiol conjugates are analyzed by monitoring the neutral loss of 129 Da arising from the cleavage of the carbon–sulfur bond of the pseudomolecular anion $[M - H]^-$.[15] In the ESI mode, a similar fragmentation is also preferred by the protonated molecular ion $[M + H]^+$ of all three conjugates of VPA (*m/z* 304) studied giving rise to the product ion *m/z* 130. The enhanced sensitivity

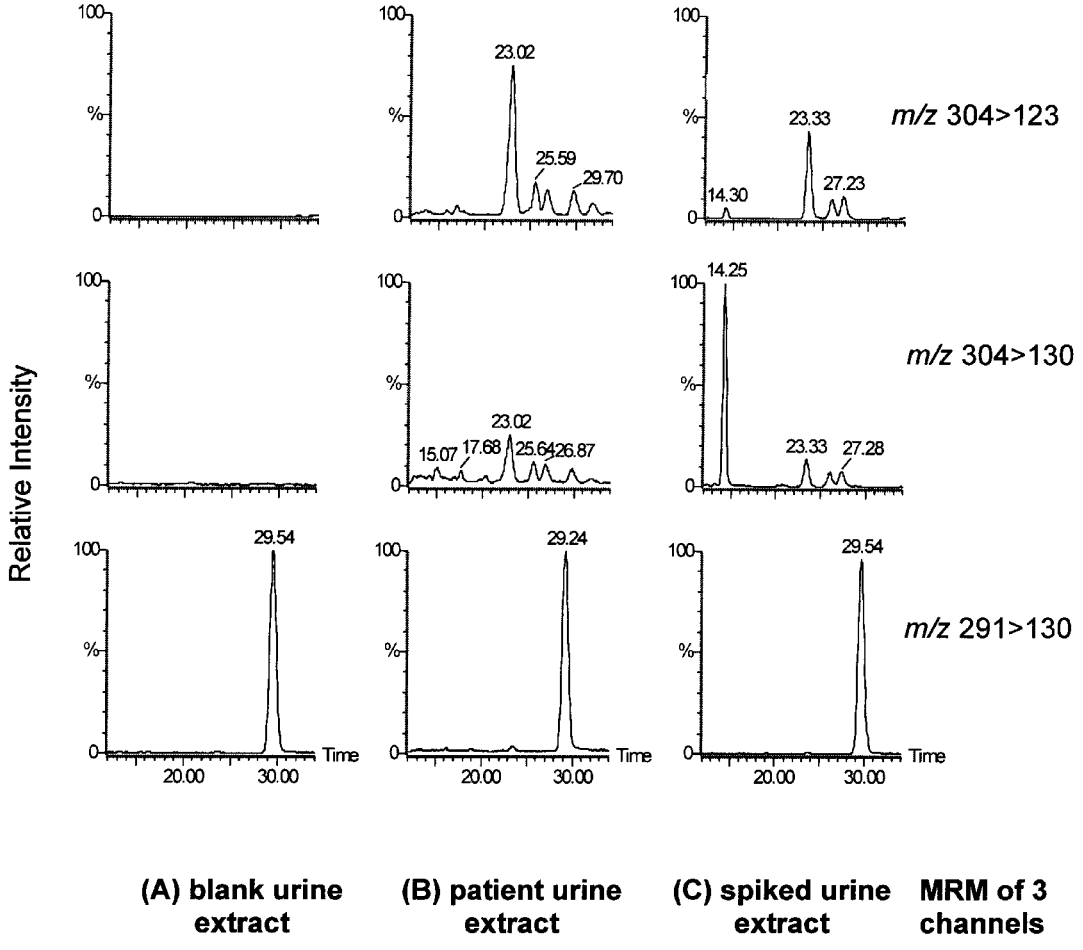

Figure 2. On-line LC/ESI-MS/MS of NAC I, NAC II and NAC III by MRM of *m/z* 304 to 123 and *m/z* 304 to 130 during a quantitation analysis. NAC III eluted at retention time $t_R = 14.30$ min, NAC II at $t_R = 23.33$ min and NAC I at $t_R = 27.23$ and 27.28 min. FVPA-GLN (IS) eluted at $t_R = 29.54$ min by MRM of *m/z* 291 to 130.

of the detector in the ESI mode allowed the accurate analysis of the conjugates by MRM of the transition *m/z* 304 to 130. For identification purposes, the transition *m/z* 304 to 123 was also monitored as described previously (S. V. Gopaul *et al*., *Drug. Metab. Dispos.*, in press) and was found equally suitable for the quantitation of NAC I and NAC II. The ionization of the $[MH]^+$ ion of FVPA-GLN under CID gives rise to *m/z* 130[20] and the transition *m/z* 291 to 130 was therefore monitored for the IS and was appropriate for the assay. Figure 2 shows the elution of the conjugates and IS during one quantitative analysis.

The GC/MS assay employed here was a modification and expansion of the assay developed earlier in this laboratory[3] where NAC I was not available in the free acid form. In the current assay, NAC I, NAC II and NAC III were all available as free acids. Contrary to the original method, this expanded assay utilized a liquid–liquid extraction procedure at pH 3–4 with H_3PO_4 to minimize the hydrolysis of the conjugates, particularly NAC IV, the IS. The extraction solvent, ethyl acetate, was appropriate for extracting all of the thiol metabolites and the IS. Derivatization of NAC I, NAC II, NAC III and NAC IV with PFBBr appeared to be complete when carried out at 50 °C for 1 h with no indication of thermal decomposition of the conjugates or the IS. Consistent with previous findings reported for NAC I and NAC IV,[3] the derivatization with PFBBr to form either the mono-PFB derivative of NAC III or the di-PFB derivatives of NAC I and NAC II and NAC IV was relatively facile.

The assay was then designed to selectively monitor *m/z* 482, 302 and 414, the $[M - 181]^-$ fragment of each ester, respectively. The PFB derivatives of the conjugates and IS produced sharp chromatographic peaks. The slow oven temperature program employed facilitated the separation of the conjugates from each other and any endogenous compounds as shown in Fig. 3. As previously reported (S. V. Gopaul *et al*., *Drug. Metab. Dispos.*, in press), the separation of the isomers of NAC I was not feasible with the relatively non-polar capillary column employed in this assay. On the other hand, the epimeric isomers of NAC III were partially resolved.

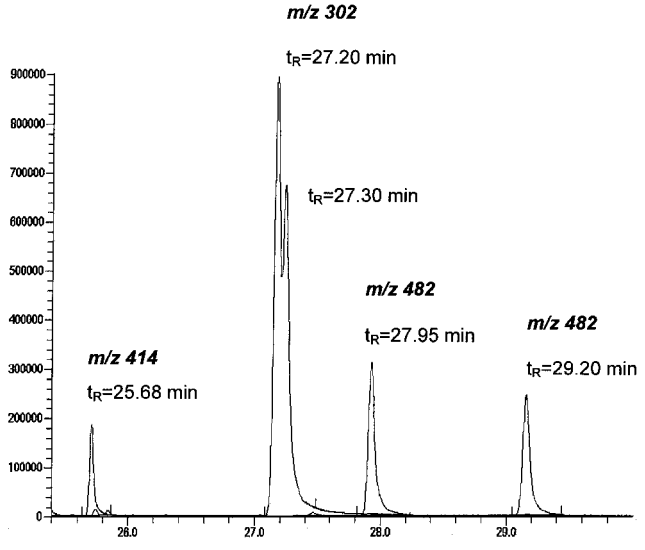

Figure 3. GC/NICI-MS SIM chromatograms of the PFB-derivatized extract of a control urine sample spiked with NAC I, NAC II, NAC III and NAC IV. The $[M - 181]^-$ carboxylate anion of the di-PFB derivatives of NAC I (*m/z* 482) eluted at $t_R = 27.95$ min, NAC II (*m/z* 482) at $t_R = 29.20$ min and NAC IV (*m/z* 414) at $t_R = 25.68$ min. The PFB derivative of NAC III isomers (*m/z* 302) eluted at $t_R = 27.20$ and 27.30 min.

The sensitivity of the GC/MS assay based on a signal-to-noise ratio (S/N) > 3 was superior to that observed for the LC/MS/MS ESI. The LOQ of all three conjugates was 0.1 μg ml^{-1} by both the LC/MS/MS and GC/MS methods. This corresponded to 1 ng of NAC I, NAC II and NAC III injected on to the HPLC column compared with 100 pg of the same compounds injected on to the GC/MS capillary column. The calibration curves were linear over the concentration ranges 0.10–1.0 μg ml^{-1} by LC/MS/MS and 0.10–5.0 μg ml^{-1} by GC/MS and afforded correlation coefficients of (r^2) of 0.999 or better for NAC I, NAC II and NAC III by both methods (Table 1). For both assays, the concentration ranges of the calibration curves were suitable for the present study. The narrower calibration range by LC/MS/MS is indicative of saturation of the detector occurring at concentrations above 1 μg ml^{-1}. This could be improved by injecting a more dilute sample on to the column.

The precision studies conducted for the assay of NAC I and NAC II showed that both methods were reproducible and reliable for quantitation within the calibration concentration ranges studied. The results for the inter- and intra-assay variations and accuracy studies of the two assays are detailed in Tables 1–3. The variation of the calibration curves of NAC I was <13% and that of NAC II was <20% based on the slopes of six and seven consecutive analytical days (Table 1), or triplicate analyses of samples spiked with known concentrations (Table 2) for both the LC/MS/MS and GC/MS assay. The intra-assay variation was <13% by both methods based on samples spiked at low and high concentrations (Table 3). In general, all quality control samples or spiked samples run in triplicate throughout the study were well within 80–120% of their expected values, indicating good accuracy (Tables 2 and 3).

The extraction recoveries of the conjugates were 50–60% by GC/MS and nearly 100% by LC/MS/MS. However, the high sensitivity of the GC/MS detector compensated for the lower recovery of the conjugates by the liquid–liquid extraction method and the overall reproducibility and accuracy of the assay remained high. Whereas the LC/MS/MS assay is more selective and can analyze both isomers of NAC I individually, the GC/MS assay can measure the conjugates over a wider concentration range. Therefore, both assays can be used individually or in complementary ways to identify and

Table 1. Inter-assay variation based on results of GC/MS calibration curves for NAC I and NAC II obtained on seven consecutive analytical days (n = 7) and LC/MS/MS calibration curves for NAC I and NAC II obtained on six consecutive analytical days (n = 6)

		NAC I		NAC II	
Method		Slope	r^2	Slope	r^2
GC/MS	Mean	0.28	0.9998	0.158	0.9990
	CV (%)	6.7	0.015	12.7	0.002
LC/MS/MS	Mean	0.17	0.9998	0.26	1.000
	CV (%)	5.9	0.001	19.5	0.003

Table 2. Inter-assay variability and accuracy based on results obtained on three analytical days (n = 3) for samples spiked at three concentrations for NAC I and NAC II by GC/MS and LC/MS/MS

Method		NAC I (μg ml^{-1})			NAC II (μg ml^{-1})		
GC/MS	Expected	0.12	0.42	0.80	0.12	0.42	0.80
	Mean	0.11	0.34	0.73	0.10	0.51	0.78
	CV (%)	0.9	12.1	5.8	6.3	4.3	16.0
	Observed (% of expected)	99	93	101	83	105	98
LC/MS/MS	Expected	0.12	0.42	0.80	0.12	0.42	0.80
	Mean	0.13	0.44	0.73	0.12	0.45	0.65
	CV (%)	8.7	9.0	8.6	7.3	8.7	12.8
	Observed (% of expected)	115	106	98	104	107	81

Table 3. Intra-assay variability and accuracy based on three replicates (n = 3) of two spiked samples of NAC I and NAC II analyzed in triplicate in one analytical day by GC/MS and LC/MS/MS

Method		NAC I (μg ml^{-1})		NAC II (μg ml^{-1})	
GC/MS	Expected	0.12	0.80	0.12	0.80
	Mean	0.11	0.74	0.12	0.74
	CV (%)	6.5	12.9	5.6	9.2
	Observed (% of expected)	94	92	108	93
LC/MS/MS	Expected	0.12	0.80	0.12	0.80
	Mean	0.12	0.91	0.13	0.93
	CV (%)	2.5	6.3	7.3	7.8
	Observed (% of expected)	103	113	110	116

quantitate the conjugates with a high degree of accuracy and selectivity.

Both assays were used to determine the recovery of VPA in the form of NAC conjugates in seven urine samples from four patients on VPA. By GC/MS, the observed concentration of the NAC conjugates ranged from 0.10 to 0.67 μg ml^{-1} for NAC I and from 0.12 to 0.73 μg ml^{-1} for NAC II. By LC/MS/MS the concentrations observed were 0.10–0.49 μg ml^{-1} for NAC I and 0.13–0.78 μg ml^{-1} for NAC II. A comparison of the results by LC/MS/MS and GC/MS (Fig. 4) for the urinary concentrations of NAC I and NAC II for the seven samples analyzed showed that there was a good correlation ($r^2 = 0.90$ and 0.91, respectively) between the two assays. The LC/MS/MS assay confirmed that both isomers of NAC I were always present in equal amounts in the urine of patients on VPA. Urine NAC II concentrations were confirmed at concentrations consistently higher than NAC I by GC/MS and LC/MS/MS.

The calculated recovery of thiol conjugates in the urine from four patients averaged 0.004–0.088% of a VPA dose by GC/MS and 0.004–0.109% of a VPA dose by LC/MS/MS (Table 4) during a 24 h period. While the amount of the conjugates recovered constituted a relatively small percentage of the VPA dose, it is important to consider that a daily dose of VPA in this study is relatively large, ranging between 375 and 1250 mg for the patients studied. Hence the results suggest that each of these patients was exposed to about 200–500 μg of reactive (*E*)-2,4-diene VPA, the apparent hepatotoxic metabolite of

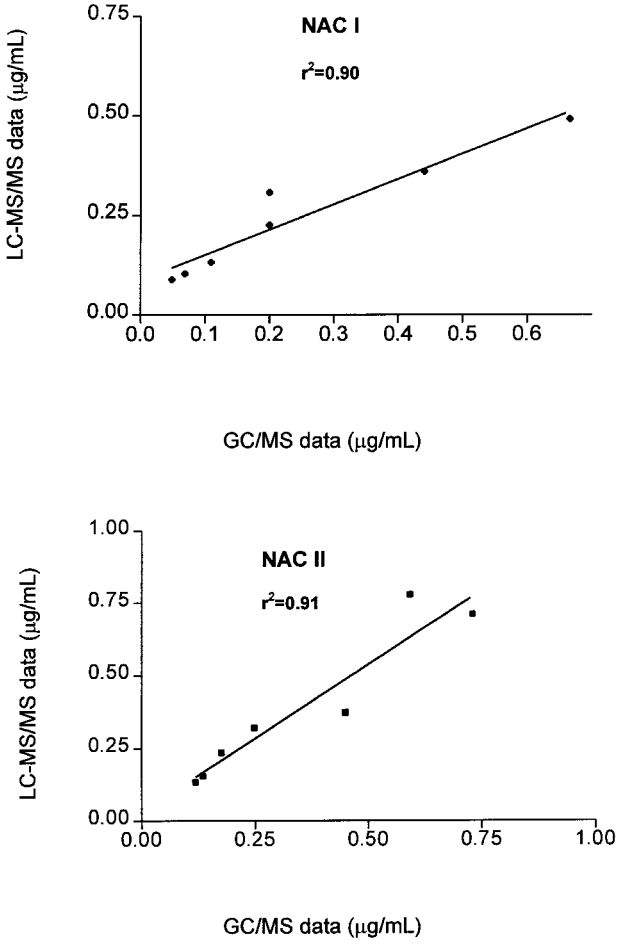

Figure 4. Graphical comparison of LC/ESI-MS/MS results versus GC/NICI-MS results obtained following the analyses of seven urine samples from patients on VPA.

VPA with each VPA dose. It can be reasoned that high-risk patients (i.e. those less than 2 years of age and on polytherapy) are exposed to relatively higher and more harmful amounts of reactive (*E*)-2,4-diene VPA, perhaps because of the higher dose and increased metabolism of VPA due to young age and enzyme induction.

Hence, to test this hypothesis, the GC/MS and LC/MS/MS assays are useful quantitative tools for the measurement of NAC I, and NAC II in biological fluids of humans and can also be utilized for other species. Since their development, the assays have been applied in the study of thiol conjugates arising from the biotransformation of 4-ene VPA in different species (unpublished results) and

Table 4. Recovery of a VPA dose as NAC I and NAC II calculated for 24 h urine samples from four patients

	Total NAC I and NAC II (% VPA)	
Patient No.	GC/MS	LC/MS/MS
1	0.088	0.109
2	0.004	0.004
3	0.012	0.018
4	0.055	0.053

to demonstrate the relationship between the NAC conjugates of (*E*)-2,4-diene VPA and the risk factors of VPA hepatotoxicity (unpublished results).

CONCLUSION

Two assays have been developed for the measurement of thiol conjugates of VPA, namely NAC I and NAC II, in human urine samples. Each assay offers unique advantages over each other, including costs and availability of equipment. Whereas the LC/MS/MS assay can measure the NAC conjugates directly as intact molecules in their free acid forms, the GC/MS assay requires the derivatization of the mercapturic acids. However, the sensitivity of the GC/MS assay for the derivatives was 10 times higher than that observed for the underivatized conjugates by LC/MS/MS.

For both assays, the precision and accuracy did not differ from each other. Both assays were reproducible and had good accuracies. The results obtained by both methodologies correlated well with each other when samples from patients on VPA were analyzed.

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