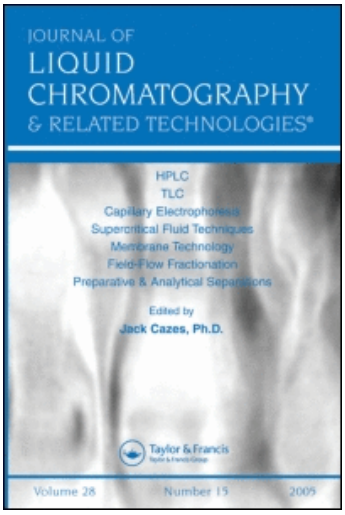

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

AN HPLC ASSAY FOR CARBAMAZEPINE PHASE I METABOLITES AND THEIR GLUCURONIDES IN URINE

D. M. Reith[a]; G. R. Cannell[a]
[a] Centre for Studies in Drug Disposition, Herston, Australia

Online publication date: 13 January 2005

To cite this Article Reith, D. M. and Cannell, G. R.(1999) 'AN HPLC ASSAY FOR CARBAMAZEPINE PHASE I METABOLITES AND THEIR GLUCURONIDES IN URINE', Journal of Liquid Chromatography & Related Technologies, 22: 12, 1907 — 1918
To link to this Article: DOI: 10.1081/JLC-100101775
URL: http://dx.doi.org/10.1081/JLC-100101775

J. LIQ. CHROM. & REL. TECHNOL., 22(12), 1907–1918 (1999)

AN HPLC ASSAY FOR CARBAMAZEPINE PHASE I METABOLITES AND THEIR GLUCURONIDES IN URINE

D. M. Reith, G. R. Cannell

Centre for Studies in Drug Disposition
Clinical Sciences Building, RBH
Herston, 4029, Australia

ABSTRACT

A binary gradient HPLC assay was developed for the separation and quantitation of carbamazepine (CBZ), carbamazepine-epoxide (CBZ-ep), carbamazepine-10,11-trans-diol (CBZ-diol), carbamazepine-2-hydroxide (CBZ-OH), carbamazepine-3-hydroxide (CBZ-3OH) and carbamazepine-acridan (CBZ-acr) extracted from patient urine. The internal standard was 10-methoxy-carbamazepine. The initial phase was 70:15:15 phosphate buffer-methanol-acetonitrile followed by a linear gradient commencing at 13 minutes to 50:35:15 phosphate buffer-methanol-acetonitrile at 24 minutes. The flow rate was constant at 2 mL/min. The UV absorbance detector wavelength was changed at 18 minutes from 240 nm to 280 nm. Typical retention times for CBZ-diol, CBZ-2OH, CBZ-ep, CBZ-3OH, CBZ-acr, CBZ and internal standard were 5.26, 8.36, 10.46, 12.51, 14.2, 23 and 27.53 minutes respectively. The minimum quantifiable limit (MQL) for all of the analytes was 0.2 μg/mL except for CBZ-2OH where the MQL was 2 μg/mL. Precision and accuracy of the assay was 1.3 to 19.4% for the 6 analytes.

Liquid-liquid extraction with ethyl acetate resulted in recovery of the analytes from urine greater than 74% except for CBZ-diol where recovery was 40%. The concentrations of the glucuronides of CBZ and metabolites were calculated by measuring their concentrations before and after hydrolysis.

INTRODUCTION

Carbamazepine (CBZ) is widely used as an anticonvulsant in adults and children and it is the most widely prescribed anticonvulsant in Australia.[1] It is extensively metabolized and the measurement of its urinary metabolites provides a convenient tool for examining metabolic pathways. HPLC methods to measure carbamazepine and metabolites in urine and plasma have been developed but these have either extended run times, poor peak definition, or are limited to 2 to 4 metabolites.[2-7] Carbamazepine and metabolites can also be measured with LC/MS and GC/MS methods but access to these systems can be limited.[8,9] In this study a reverse phase gradient HPLC method was developed for measuring the levels of carbamazepine (CBZ), carbamazepine-10,11-epoxide (CBZ-ep), carbamazepine-10,11-trans-diol (CBZ-diol), carbamazepine-2-hydroxide (CBZ-2OH), carbamazepine-3-hydroxide (CBZ-3OH), carbamazepine acridan (CBZ-acr) and their glucuronides in urine (Figure 1).

EXPERIMENTAL

The HPLC system included a Waters 600 quadratic pump, a Waters 600E system controller, a Waters WISP 710 autosampler, a Waters Radial-PAK C18 column, a Waters 486 tunable UV absorbance detector, and a Shimadzu CR3-A integrator. Following development of the assay the detector output was integrated using a Millenium version 2.12 software package with windows for workgroups version 3.11 as the operating system.[10] Peaks were integrated visually and quantitation was performed using standard curves calculated by the software.

CBZ was obtained from Sigma Chemical Company (St Louis, MO., USA). CBZ-EP, CBZ-diol, CBZ-2OH, CBZ-3OH and CBZ-acr were obtained from Ciba-Geigy (Basel, Switzerland). The internal standard, 10-methoxy carbamazepine, was also obtained from Ciba-Geigy. β-Glucuronidase was supplied by Sigma. HPLC grade methanol and acetonitrile were obtained from Mallinckrodt (Paris, KY., USA). Reagent grade 18 megaohm water was obtained by filtering reverse osmosis pretreated water through a Barnstead Nanopure II filtration unit. Phosphate buffers were made by combining

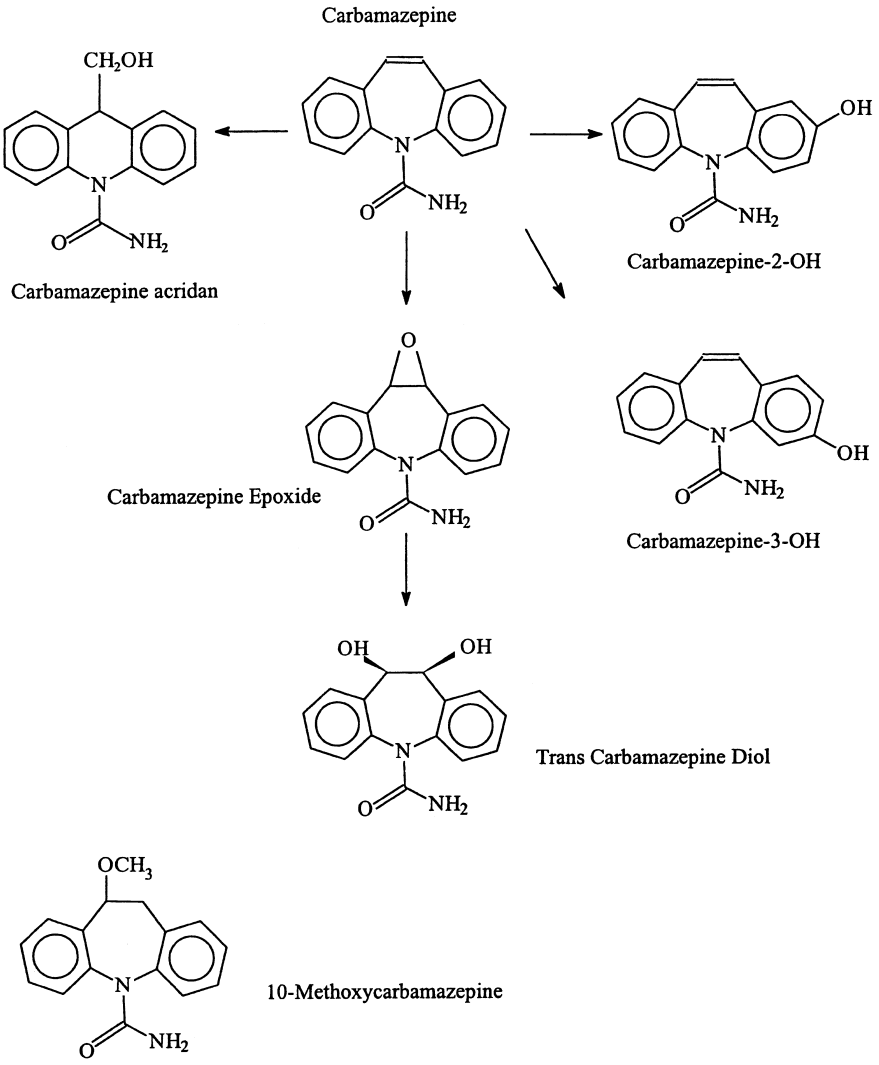

Figure 1. Metabolism of carbamazepine.

potassium dihydrogen phosphate (0.067 M) with disodium phosphate (0.067 M) to produce two solutions with a pH of 5.0 and 7.0 respectively. The pH 7.0 phosphate buffer was diluted 1:6 with water to make up the phosphate buffer used in the mobile phase (0.0095 M). Glycine buffer was made by combining a solution of glycine (0.1 mM) and NaCL (0.1 mM) with NaOH (0.1 mM) to produce a solution with a pH of 12.5.

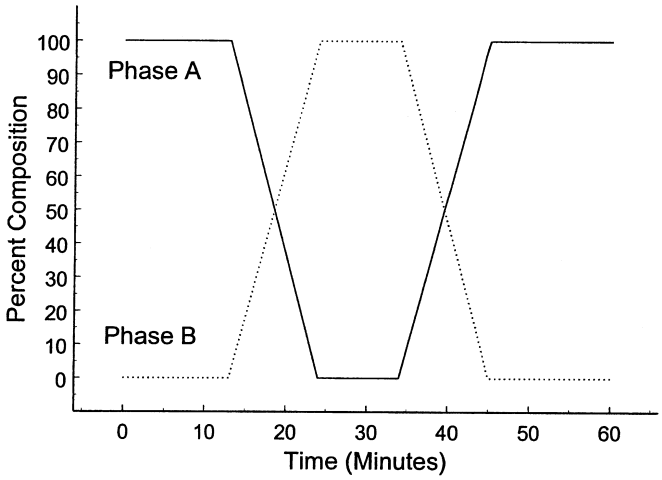

Figure 2. Solvent gradient.

Stock solutions (200 μg/mL) of carbamazepine and each metabolite were prepared in methanol. A working mixture was prepared by combining aliquots of each stock solution to give a final concentration of 25 μg/mL for each analyte. The internal standard was prepared at a concentration of 25 μg/mL separately in methanol. Aliquots (100 μL) of the stock solutions of analytes and internal standard were diluted with 300 μL of methanol. A sample (20 μL) of this mixture was analyzed by HPLC.

To test separation of CBZ and its metabolites a solvent gradient was developed by combining water, phosphate buffer, methanol, and acetonitrile in differing amounts in a sequential manner. When the optimal separation was obtained phosphate buffers from pH 5 to pH 7.5 in pH 0.5 increments were individually tested to obtain the best separation and peak shape. Mobile phases were degassed and filtered using a Waters solvent filtration unit with a 0.5 μm filter. The phases were continuously sparged with helium at a flow rate of 5 mL per minute. Two phases were used in the final gradient. Phase A consisted of 70% phosphate buffer (pH 7, 0.0095 M), 15% methanol, and 15% acetonitrile. Phase B consisted of 50% phosphate buffer (pH 7, 0.0095 M), 35% methanol and 15% acetonitrile. The gradient is represented in Figure 2. The flow rate was constant at 2 mL/min for the full 60 minutes of each run. The detector wavelength was 240 nm from 0 to 18 minutes and 280 nm from 18 to 40 minutes. One hundred percent phase A was pumped at a flow rate of 2 mL/min through the system for the last 15 minutes of each run to allow equilibration.

Patient urine samples were collected and pH adjusted to 5 prior to freezing and storage at -20°C. Samples were thawed for analysis in a water bath at 37°C and 500 μL was added to test tubes containing 80 μL internal standard and 1 mL phosphate buffer pH 5. This step was repeated and the second urine sample was incubated with 1000 U β-glucuronidase for four hours at 37°C. Glycine buffer (100 μL) and ethyl acetate (3 mL) were added to each tube and mixed by vortexing. The tubes were then centrifuged for 5 minutes at 2500 rpm and 2 mL of the supernatant was transferred to clean test tubes and evaporated to dryness under a gentle jet of nitrogen. The samples were reconstituted with 200 μL mobile phase A. A 100 μL aliquot of each sample was injected for analysis.

Standard curves were constructed from standards of each analyte prepared in concentrations of: 1000 μg/mL, 500 μg/mL, 250 μg/mL, 25 μg/mL, 5 μg/mL, 2.5 μg/mL, and 0.1 μg/mL. Forty microliters of each standard was added to 500 μL of blank urine and extracted following the same method applied to the study samples. The recovery of each analyte was calculated by extracting urine samples spiked with CBZ and metabolites to concentrations of 0.2, 2, and 40 μg/mL and quantifying against an unextracted standard curve. Recovery of internal standard was determined by assaying blank urine samples containing internal standard, CBZ and metabolites and comparing these results with those obtained by addition of the internal standard after the extraction procedure. Recovery was assessed for the pH range of 5.0 to 7.5. The optimal pH was further assessed with the addition of 3 M NaCl.

Samples of urine spiked with compounds which are likely to be present in the urine of patients with epilepsy were extracted and analyzed using the final method. The compounds were assessed at the concentrations expected at normal therapeutic ranges.

Intra-assay precision and accuracy were determined by assaying 6 replicates of blank urine containing CBZ, metabolites, and internal standards at concentrations of 0.2, 2, and 40 μg/mL against a standard curve. Inter-assay precision and accuracy were determined from the back-calculations of the standard curves and quality controls (2 μg/mL) for 6 separate runs. Precision and accuracy were calculated using the formulae:

$$\text{Precision} = \frac{\text{Standard deviation x } 100}{\text{mean}}$$

$$\text{Accuracy} = \frac{100}{n} \sum \frac{\text{Calculated Urine Concentration} - \text{Expected Urine Concentration}}{\text{Expected Urine Concentration}}$$

where n = number of replicate analyses. The lower limit of detection was defined as the lowest concentration at which both precision and accuracy were less than 20% for each analyte separately.

The coefficient of determination (r^2) was calculated for the standard curve and the percentage deviation of the quality controls (2 μg/mL) for each analyte were calculated. Ruggedness was assessed by dismantling and reassembling the apparatus on three separate occasions, separated by 6-12 months, by the same analyst employing different Waters Radial-PAK C18 columns.

RESULTS

In this analytical system, a binary mobile phase analysis was developed to separate the sample mixture of CBZ and 5 metabolites. The initial wavelength of 240 nm was required because of the poor absorbance of CBZ-ep at higher wavelengths and the final wavelength was used to reduce interference from endogenous compounds in the urine. Representative chromatograms are presented as Figures 3 and 4 showing analysis of representative blank urine and urine containing CBZ and metabolites respectively. The retention times in a typical assay were: CBZ-diol 5.26 minutes (coefficient of variability 0.36), CBZ-2OH 8.36 (0.54), CBZ-ep 10.46 (0.5), CBZ-3OH 12.51 (0.59), CBZ-acr 14.2 (0.55), CBZ 23.0 (0.17), and methoxy-CBZ 27.53 (0.09).

Baseline separation of p-OH-phenytoin and CBZ-2OH was not achieved with retention times of 7.9 and 8.36 respectively. Phenobarbitone eluted at 6.5 minutes but did not interfere with quantitation of CBZ-diol (5.26 min) or CBZ-2OH (8.36 min) because of baseline separation. The following compounds did not interfere with the assay: ethosuximide, methsuximide, gabapentin, tiagabine, lamotrigine and phenytoin. Vigabatrin and valproate were not tested for interference because of polarity and lack of absorbance at the wavelengths used respectively. There was no interference from caffeine or its major metabolites.

Calculated precision and accuracy results for CBZ and its metabolites are presented in Table 1. The minimum quantifiable limit (MQL) for all the analytes was 0.2 μg/mL except for CBZ-2OH where the MQL was 2 μg/mL. The mean r^2 for each of the analytes was between 0.998 and 0.9991. Table 2 presents coefficients of determination, slopes and intercepts for each of the analytes. Recoveries of each analyte were: CBZ-diol 40%, CBZ-2OH 90%, CBZ-ep 74%, CBZ-3OH 96%, CBZ-acr 86%, and CBZ 101%. Recovery of internal standard was 70%. Recovery was optimal at a pH of 7 and a final concentration 0.2 M sodium chloride in samples did not improve recovery further.

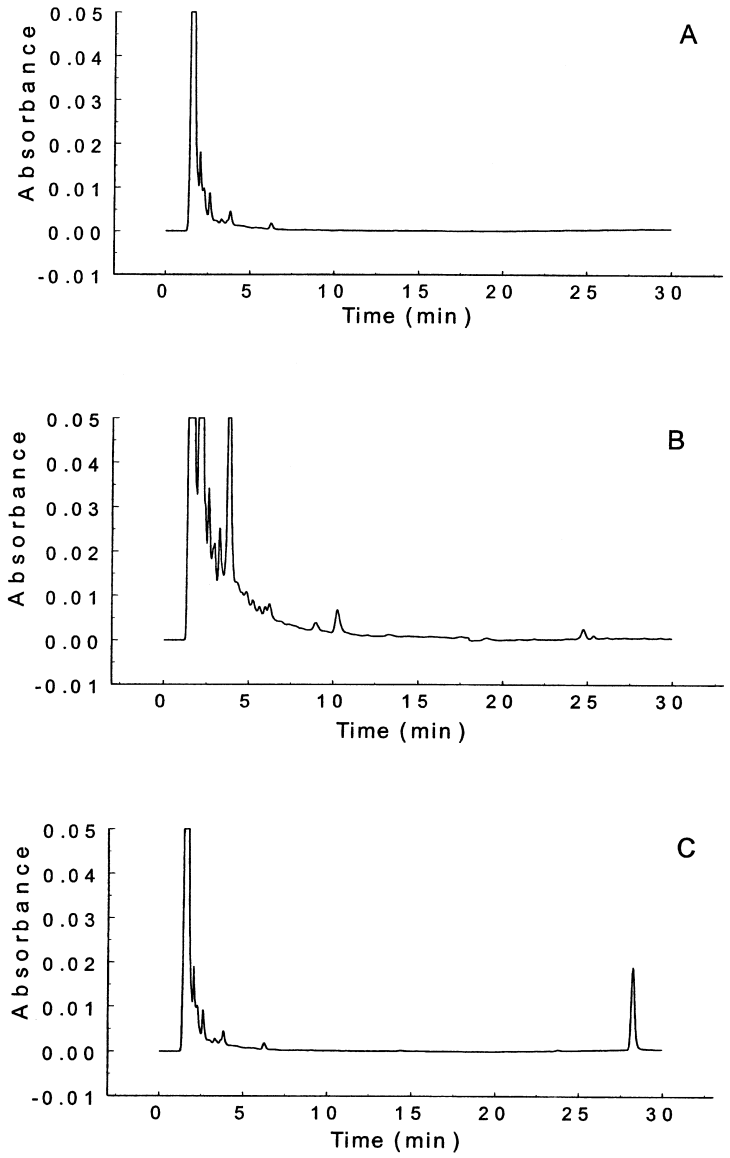

Figure 3. HPLC separation of (A) extracted blank urine, (B) extracted hydrolyzed blank urine and (C) extracted blank urine with internal standard.

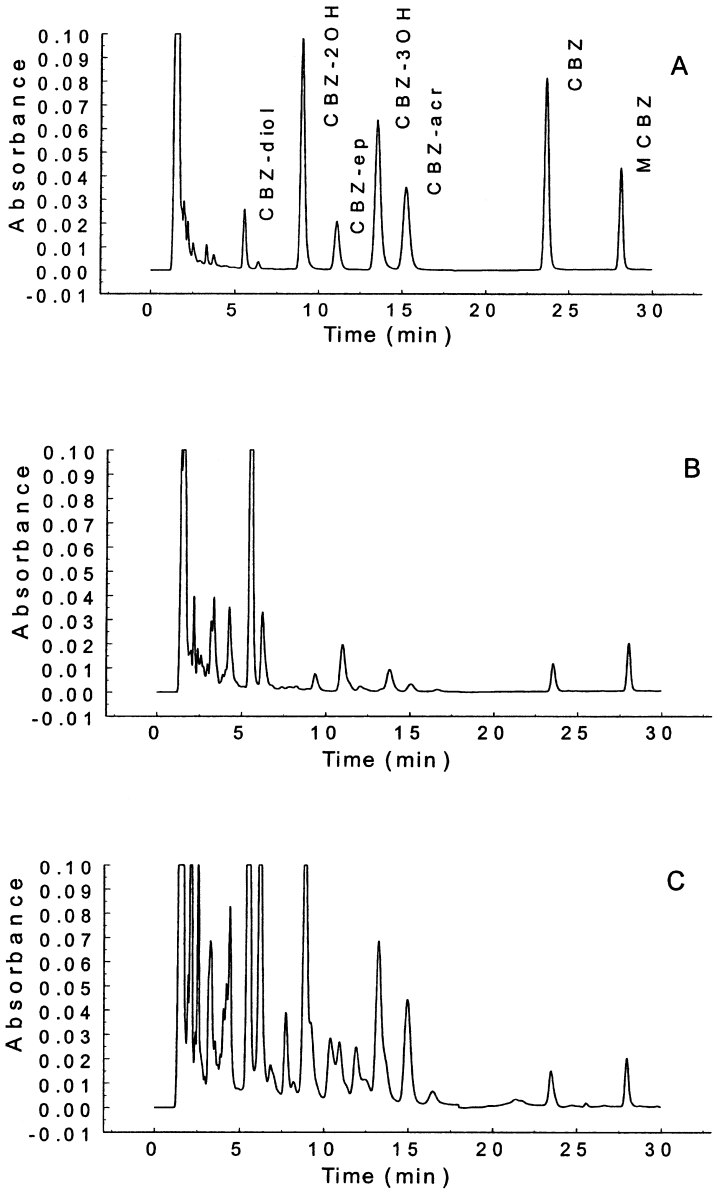

Figure 4. HPLC separation of (A) extracted standards, (B) extracted patient urine and (C) extracted hydrolyzed patient urine.

Table 1

Intra-Assay (n=6) and Inter-Assay (n=6) Precision and Accuracy

Concentration		Intra-Assay		Inter-Assay	
μg/mL	nmol/L	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
CBZ-Diol					
0.2	0.74	5.7	6.7	15.3	13.8
2	7.4	4.9	5	9.3	12.2
40	147.99	5.7	4.3	1.8	2.9
CBZ-2OH					
0.2	0.79	3.3	5	46.3	119
2	7.93	3.3	3.8	12.4	9.9
40	158.6	6.7	4.9	3.6	3.6
CBZ-ep					
0.2	0.79	1.6	1.3	5.7	19.4
2	7.93	3	3.2	10.4	14.7
40	158.56	6.2	4.7	2.2	1.8
CBZ-3OH					
0.2	0.79	3.8	5.7	7	13.6
2	7.93	2.8	4.8	7.8	10
40	158.56	6.7	5.2	2.4	2.4
CBZ-acr					
0.2	0.79	2.6	3.3	10.4	6.6
2	7.93	5.9	5	6.9	5.4
40	158.56	6.1	4.9	2.5	2.4
CBZ					
0.2	0.85	2.8	6.6	4.6	7.7
2	8.46	5.1	4.9	4.4	3.1
40	169.3	6.8	5.4	2.5	3

Table 2

Linearity of Assay of CBZ and Metabolites

	r^2 (Mean)	a (Range)	b (Range)
CBZ-diol	0.998	-0.001 to 0.0214	0.1681 to 0.197
CBZ-2OH	0.9989	-0.02 to 0.0096	1.2786 to 1.479
CBZ-ep	0.9987	-0.006 to -0.0017	0.2807 to 0.3412
CBZ-3OH	0.9988	-0.002 to -0.0326	1.2444 to 1.3584
CBZ-acr	0.9991	-0.002 to 0.0133	0.7146 to 0.7829
CBZ	0.9991	-0.002 to 0.0133	1.1658 to 1.2747

DISCUSSION

A specific and reproducible assay for the measurement of CBZ, CBZ-ep, CBZ-diol, CBZ-2OH, CBZ-3OH, CBZ-acr was developed. The concentrations of the glucuronides of the analytes were also measured by analyzing duplicate samples after hydrolysis. Other assays have been described for measuring these metabolites but they either require LC/MS or GC/MS methodology or are isocratic HPLC methods which have peaks still eluting at 60 minutes.[3,8,9] The use of a gradient method also enables the clearing of the column of interfering, late eluting peaks from endogenous compounds present in urine.

In this analysis there was minimal interference to the compounds measured from endogenous compounds excreted in urine. Analysis of samples before and after hydrolysis with β-glucuronidase enabled calculation of free and conjugated CBZ and metabolites.

CBZ-ep was quantitated at 240 nm as it was the only metabolite which absorbed poorly at 280 nm. The ability to change wavelengths to quantitate CBZ and internal standard at 280 nm enabled a decrease in potential interference from endogenous compounds with the later peaks.

A number of unknown peaks were present in the patient urine that were not present in blank urine. These peaks, which may represent some of the putative 33 metabolites of CBZ,[8] were not identified due to the absence of standard material. Anticonvulsants typically prescribed in combination with carbamazepine were assessed for interference and with the exception of p-OH-phenytoin did not interfere with the assay. As p-OH-phenytoin is a major metabolite of phenytoin, the measurement of CBZ-2OH would not be reliable in a patient taking phenytoin in addition to CBZ.

Recovery of the analytes from urine was 74% or greater with the exception of CBZ-diol with a recovery of 40%. CBZ-diol is the most polar of these compounds but despite this the recovery of CBZ-diol was consistent.

Bonato, *et al.* reported that the addition of sodium chloride improved recovery from plasma but, in the present study, addition of sodium chloride did not improve recovery of CBZ or its metabolites from urine.[5]

ACKNOWLEDGMENTS

The authors wish to acknowledge Ciba-Geigy for supplying CBZ metabolite standards. Dr Reith was supported by Royal Children's Hospital Foundation grant number 376.

REFERENCES

1. P. McManus, 1997 Drug Utilization Sub-Committee Data (1998).

2. R. D. Chelberg, S. Gunawan, D. Treiman, Ther Drug Monit.,**10(2)**, 188-193 (1988).

3. I. Bernus, W. D. Hooper, R. G. Dickinson, M. J. Eadie, Epilepsy Res., **21**, 65-75 (1995).

4. A. S. Elyas, N. Ratnaraj, V. Goldberg, P. Lascelles, J. Chromatogr., **231**, 93-101 (1982).

5. R. S. Bonato, V. Lanchote, D. de Carvalho, J Anal Toxicol., **16**, 88-92 (1992).

6. L. Lanchote, P. S. Bonato, G. M. Campos, I. Rodrigues, Ther Drug Monit., **17**, 47-52 (1995).

7. G. W. Mihaly, J. Phillips, W. Louis, F. Vajda, Clin Chem., **23/12**, 2283-2287 (1977).

8. K. Lertratanangkoon, M. Horning, Drug Metab. Dispos., **10(1)**, 1-10 (1982).

9. L. Maggs, M. Pirmohamed, N. R. Kitteringham, B. K. Park, Drug Metab Dispos., **25(3)**, 275-280 (1997).

10. **Waters Millenium Chromatography Manager**, 2.12 ed., Waters Corp. Milford, MA, 1995.

Received July 3, 1998
Accepted September 15, 1998
Manuscript 4856

Request Permission or Order Reprints Instantly!