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A capillary gas chromatographic assay with nitrogen phosphorus detection for the quantification of topiramate in human plasma, urine and whole blood

J.M. Riffitts, L.G. Gisclon, R.J. Stubbs *, M.E. Palmer

Department of Drug Metabolism, The R.W. Johnson Pharmaceutical Research Institute, Welsh and McKean Roads, Spring House, PA, 19477, USA

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Abstract

An accurate and robust method involving liquid–liquid extraction and capillary gas chromatographic (GC) assay with nitrogen phosphorus detection (NPD) was developed and validated for the quantitative determination of topiramate [2,3:4,5-bis-*O*-(-1-methylethylidene)-β-D-fructopyranose sulfamate], Topamax™, an anticonvulsant drug, in human plasma, urine, and whole blood. The galactopyranose analog of topiramate was used as the internal standard. A DB-5, fused silica capillary column (J&W Scientific, Folsom, CA) was used, yielding typical retention times of 4.95 min for topiramate and 5.32 min for the internal standard in human plasma. The assay involved organic extraction with methyl *t*-butyl ether (MTBE) from base, a back extraction into acid and a second extraction in MTBE. The organic solvent was evaporated, and the residue was redissolved and injected for analysis. The standard curve was validated from 0.5 to 50 μg ml^{-1} for human plasma and whole blood, and from 1.0 to 50 μg ml^{-1} for urine. Peak area ratios of drug to internal standard were determined and used to construct a standard curve. The resulting chromatograms showed no endogenous interfering peaks with the respective blank human fluids. Chromatograms corresponding to topiramate and the internal standard produced sharp peaks that were well resolved. This assay showed precision and accuracy of $\leq 5\%$. Two minor human metabolites of topiramate did not interfere with the assay. This assay was successfully applied to determine the pharmacokinetics of topiramate during the development of this drug.

Keywords: Topiramate; Gas chromatography; Human plasma; Human urine; Human whole blood

1. Introduction

Topiramate: [2,3:4,5-bis-*O*-(1-methylethylidene)-β-D-fructopyranose-sulfamate], Topamax™, (Fig. 1) is a novel compound which has been shown to be an effective anticonvulsant with a good safety profile after oral administration in humans and animals [1]. It was developed by the R.W. Johnson Pharmaceutical Research Institute, Spring House, PA. Topiramate has been approved for the use as adjunctive therapy in pa-

* Corresponding author. Tel.: +1 2155404706; fax: +1 2156287822.

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tients with partial onset seizures with or without secondarily generalized seizures [2–4], and a reliable method of analysis was needed to support clinical studies.

Previously, gas chromatography with FID detection was used as the method of analysis for topiramate in biological fluids which gave nonspecific detection and more endogenous peaks in the chromatograms. [5] The current analytical method used is capillary gas chromatography with nitrogen phosphorus detection (GC-NPD). Prior to the GC-NPD assay, topiramate (pK_a 8.61) was selectively extracted from human biological samples under acidic (pH 4) conditions into methyl *t*-butyl ether. This was followed by back extraction into base followed by a further solvent extraction following acidification. The additional extraction step was found to be necessary to produce clean chromatograms. The recovery of topiramate was not adversely affected by the additional extraction step.

Topiramate binds to human erythrocytes, and an assay for whole blood was needed in addition to the plasma assay. A urine assay was also developed to quantify the excretion of drug by this route of elimination. Both of these procedures are described in addition to the plasma assay.

2. Experimental

2.1. Materials

Topiramate, and the internal standard 1,2:3,4-bis-*O*-(1-methylethylidene)-*β*-D-galacto-pyranose sulfamate, were obtained from the R.W. Johnson Pharmaceutical Research Institute, Spring House, PA. Methanol, and toluene, both of nanograde quality were obtained from Mallinckrodt, Paris, NY. High-purity methyl *t*-butyl ether (MTBE) was obtained from Baxter Healthcare, McGraw Park, IL. Water was deionized by reversed-phase osmosis and purified by a Milli-Q Millipore purification system (Millipore, Marlborough, MA). Sodium hydroxide and phosphoric acid 85% were certified ACS grade from Fisher, Fair Lawn, NJ; ammonium dihydrogen phodphate monobasic was HPLC grade and was also obtained from Fisher. The 0.9% sodium chloride Inj., USP, used in whole blood analysis, was obtained from Abbott Laboratories, North Chicago, IL. Control blank heparinized human plasma and whole blood were obtained from Biological Speciality, Lansdale, PA. Blank urine was obtained from male laboratory personnel.

2.2. Equipment

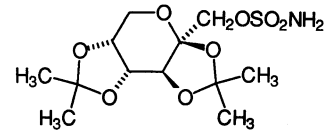
The gas chromatograph used in this assay was a Hewlett Packard 5890 Series 11 gas chromatograph with nitrogen phosphorus detection, Hewlett Packard 7673 auto-injector, and Hewlett Packard 3396A integrator.

2.3. Chromatographic conditions (GC)

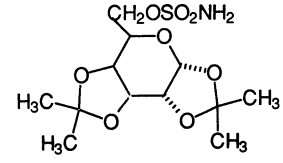
A DB-5 fused silica column, (J&W Scientific) with dimensions of 15 m × 0.32 mm I.D. and 0.25 μm film thickness was used.

2.3.1. Plasma and urine

The GC temperature profile was as follows: oven temperature equilibration time, 0.50 min; initial value, 165°C; initial time, 0.30 min; level 1 program rate, 9.0°C min^{-1}; and final value, 240°C. The injection mode was splitless, with purge. The injector temperature was 250°C and



Topiramate



Internal standard

Fig. 1. Chemical structure of topiramate and the internal standard.

the injection volume was 2.0 μl. The detector temperature was 285°C. Helium was used as the carrier gas at a flow rate of 3 ml min^{-1}.

2.3.2. Whole blood

The same initial temperature profile as plasma was used. However, a subsequent level two program rate was added which was: 30°C min^{-1}, with a final value of 290°C and final time of 2.00 min.

2.4. Preparations of standard solutions

2.4.1. Plasma and urine

An internal standard stock solution of 1 mg ml^{-1} was prepared by accurately weighing ~15 mg of the internal standard and diluting with the appropriate volume of 50% methanol in water (v/v), (internal standard stock solution). Ten millilitres of internal standard stock solution were then diluted to 200 ml with 50% methanol in water (v/v), (internal standard working solution) and mixed well. A standard stock solution of 1 mg ml^{-1} was prepared by weighing ~10 mg topiramate and diluting with the appropriate volume of 50% methanol in water (v/v). Standard solutions were prepared by using the standard stock solution and making dilutions with 50% methanol in water (v/v). All solutions were determined to be stable for at least 2 weeks when stored in the refrigerator. Standard curves were prepared by adding 50 μl of the appropriate standard and internal standard solutions to the respective human biological fluid to give final concentrations of 0.5, 1, 2, 4, 5, 10, 20, 40 and 50 μg ml^{-1} topiramate for plasma and whole blood, and 1, 2, 4, 5, 10, 20, 40 and 50 μg ml^{-1} topiramate for urine.

2.4.2. Whole blood

The 1 mg ml^{-1} internal standard stock solution prepared for plasma was used for whole blood analysis. Ten millilitress of this solution were diluted with 200 ml of 0.9% sodium chloride Inj., USP solution (internal standard solution, whole blood), and mixed well. A standard stock solution of 1 mg ml^{-1} was prepared by weighing ~10 mg topiramate and dissolved with 0.5 ml methanol.

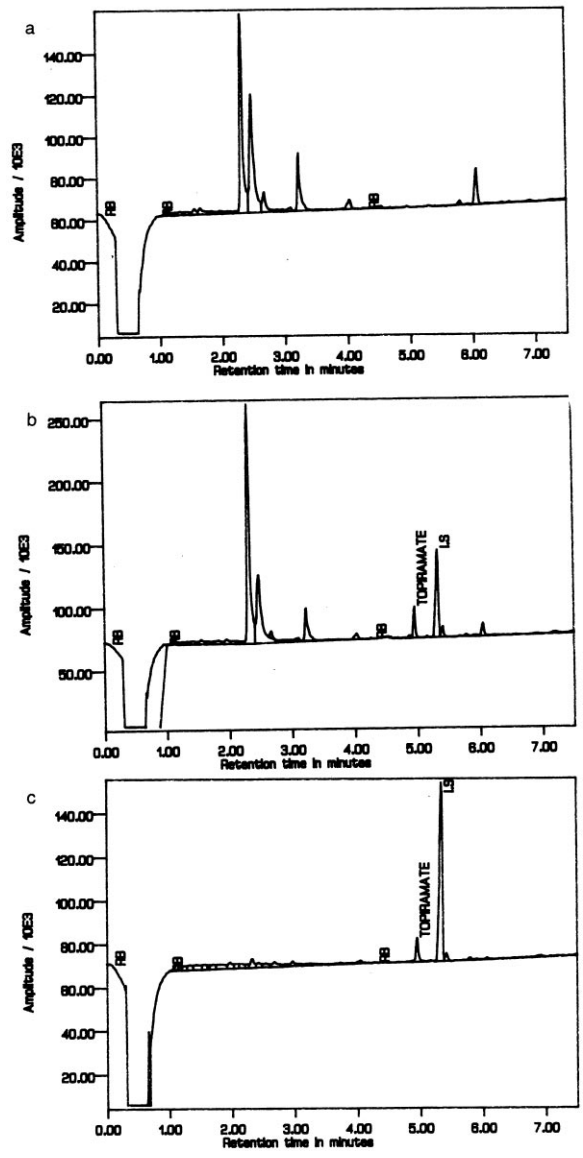


Fig. 2. Chromatograms of topiramate in human plasma: (a) blank human plasma, (b) human plasma spiked with 5 μg ml^{-1} topiramate, and (c) human plasma patient sample, 2.04 μg ml^{-1} topiramate.

An appropriate volume of 0.9% sodium chloride Inj., USP solution was added to give 1 mg ml^{-1} solution (standard stock solution, whole blood). Standard solutions were prepared by using this standard stock solution and all dilutions were made with 0.9% sodium chloride Inj., USP solu-

tion. All of these solutions were stable for at least 2 weeks when stored in the refrigerator. Standard curves were prepared by adding 50 μl of the

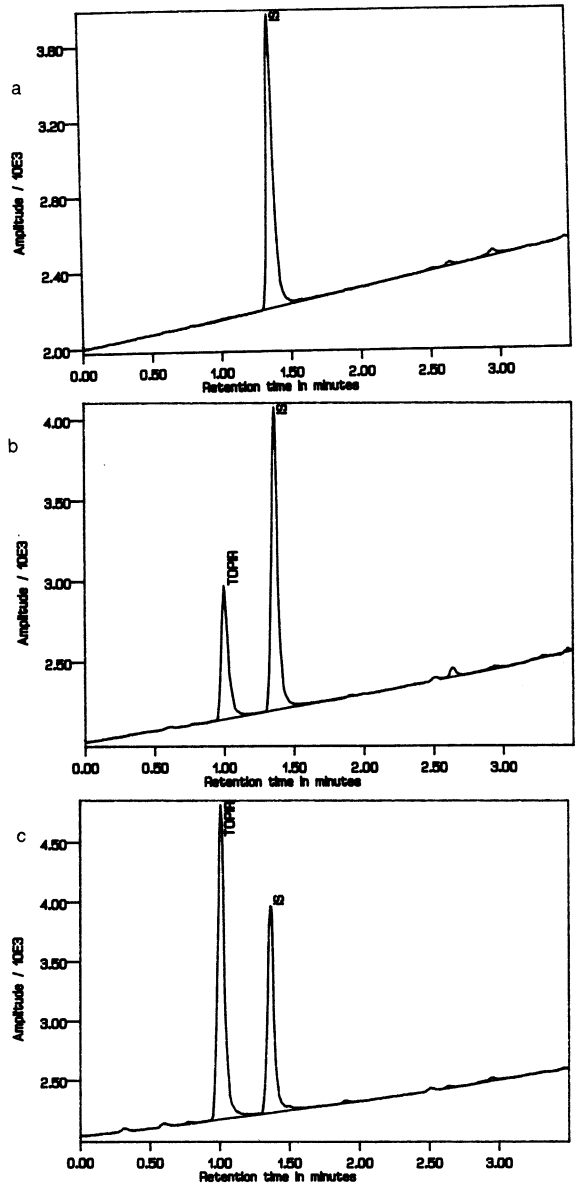


Fig. 3. Chromatograms of topiramate in human urine: (a) blank human urine, (b) human urine spiked with 10 μg ml^{-1} topiramate, and (c) human urine patient sample, 33.18 μg ml^{-1} topiramate.

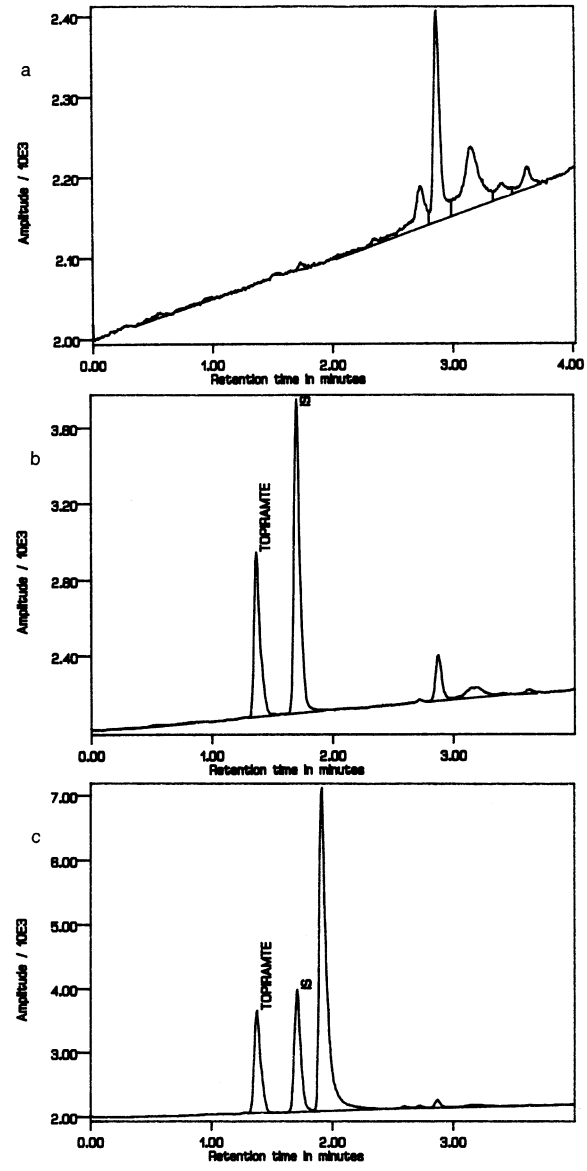


Fig. 4. Chromatograms of topiramate in human blood: (a) blank human whole blood, (b) human whole blood spiked with 10 μg ml^{-1} topiramate, and (c) human whole blood patient sample, 16.39 μg ml^{-1} topiramate.

appropriate standard and internal standard solution to whole blood to give final concentrations in the ranges of 0.5 to 50 μg ml^{-1} topiramate.

Table 1
Summary of standard curve validation for topiramate in human plasma ($n = 6$)

Concentration (μg ml^{-1})	0.50	1.00	2.00	4.00	5.00	10.0	20.0	40.0	50.0
Day 1 Line 1	0.51	1.00	1.88	4.09	5.14	10.1	20.0	39.9	50.0
Day 1 Line 2	0.52	0.98	1.91	4.08	4.90	9.6	20.8	41.2	51.8
Day 2 Line 1	0.50	1.00	2.01	4.00	5.04	10.3	20.3	39.2	48.5
Day 2 Line 2	0.51	0.94	2.09	3.94	5.40	10.0	20.0	38.6	49.7
Day 3 Line 1	0.49	1.03	2.02	4.06	5.08	10.0	20.2	39.1	48.3
Day 3 Line 2	0.50	0.97	2.06	4.18	5.15	10.1	20.2	39.3	47.0
Mean	0.50	0.99	2.00	4.06	5.12	10.0	20.2	39.6	49.1
S.D.	0.01	0.03	0.09	0.08	0.16	0.25	0.28	0.09	1.64
% C.V.	2.02	3.26	4.27	2.03	3.22	2.47	1.37	2.26	3.33
% Deviation	0.42	−1.21	−0.18	1.50	2.41	0.14	1.21	−1.07	−1.76

2.5. Quality control samples

2.5.1. Plasma

Quality control samples were prepared at concentrations 1.0, 4.0, 10, and 40 μg ml^{-1} topiramate. Approximately 10 mg of topiramate was accurately weighed and dissolved in methanol to give a 1 mg ml^{-1} solution (QC stock 1). Other QC solutions were prepared by using QC stock 1. Quantity control plasma pools (50 ml) were made by fortifying blank plasma with the appropriate QC solution to yield final concentrations of 1.0, 4.0, 10 and 40 μg ml^{-1} topiramate. These quality control samples were stored in polypropylene tubes (Sarstedt, Pennsauken, NJ) at −20°C. These quality controls were analyzed during assay validation and with daily standard curves during sample analysis.

2.5.2. Urine

Quality control samples were prepared at 3.0, 20 and 40 μg ml^{-1} topiramate following the same procedures used for plasma.

2.5.3. Whole blood

Quality control samples were prepared at 2.0, 12 and 25 μg ml^{-1} topiramate. Approximately 10 mg of topiramate was weighed and dissolved with 0.5 ml methanol. An appropriate volume of 0.9% sodium chloride Inj., USP solution was added to give a 1.0 mg ml^{-1} solution (QC stock solution, whole blood). Other QC solutions were prepared by using the QC stock solution, whole blood. Quality control whole blood pools (50 ml) were made by fortifying blank whole blood with the appropriate QC solution to yield final concentrations of 2.0, 12, and 25 μg ml^{-1} topiramate. These quality control samples were stored in polypropylene tubes at −20°C. These QC samples were also analyzed during assay validation and with daily standard curves during sample analysis.

2.6. Sample preparation

2.6.1. Plasma

An aliquot of 100 μl of each plasma sample, blank plasma, or QC sample was placed in a 10 ml conical tube. The following were added to each tube: 50 μl of internal standard solution, 50 μl 50% methanol in H_2O (v/v), (for whole blood, 50 μl of 0.9% sodium chloride Inj., USP solution was added instead), or appropriate standard solution, 250 μl of 0.1 M ammonium dihydrogen phosphate monobasic buffer, and 5 ml of MTBE. The tube was vortexed for 2 min on a mechanical vortexer (Baxter Scientific Products multi-tube vortexer), and centrifuged (Beckman GS-6R centrifuge) for 5 min at 1500 rpm. The aqueous layer was then frozen on a bed of dry ice. The ether layer was decanted into a 10 ml conical centrifuge tube. The aqueous was discarded. A volume of 500 μl of 0.2 M sodium hydroxide was added, the tube was vortexed for 2 min and centrifuged at 1500 rpm for 5 min. The organic layer was aspirated, and 300 μl of 0.2 M phosphoric acid was

Table 2
Topiramate plasma concentrations in frozen quality control samples ($n = 6$)

Theoretical concentration (μg ml^{-1})	1.00	4.00	10.0	40.0
Day 1 Line 1	1.03	4.09	10.0	37.2
Day 1 Line 2	1.05	4.26	10.1	41.6
Day 2 Line 1	1.01	4.35	10.7	40.2
Day 2 Line 2	1.09	4.47	10.3	40.3
Day 3 Line 1	1.08	4.24	10.8	40.1
Day 3 Line 2	0.96	4.25	10.8	42.3
Mean calculated conc. (μg ml^{-1})	1.04	4.28	10.5	40.3
S.D. from mean	0.05	0.13	0.36	1.75
% Acceptance	15.0	15.0	15.0	15.0
% C.V.	4.40	3.00	3.40	4.34
% Deviation from theoretical	3.61	6.95	4.58	0.65

added to the aqueous layer and vortexed for 10 s. A volume of 500 μl of 0.1 M ammonium dihydrogen phosphate monobasic buffer and of 5 ml of MTBE were added to the tube. The tube was vortexed for 2 min and centrifuged for 5 min at 1500 rpm. The aqueous layer was frozen on a bed of dry ice, and the organic layer was decanted into a 10 ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen in a heated water bath at 50–60°C. The aqueous layer was discarded and the samples were reconstituted in 150 μl of 10% methanol in toluene (v/v), vortexed for 5 s and transferred into glass autosampler vials. A 2 μl sample volume was injected onto the gas chromatograph.

2.6.2. Urine

Human urine (100 μl) may be substituted for human plasma (100 μl) and extracted and assayed for topiramate using the same procedure described for plasma.

2.6.3. Whole blood

Whole Blood was assayed using the procedure for plasma with a modified technique for pipetting blood samples. The pipette tip was rinsed with the blood sample prior to the 100 μl sample that was aliquoted for analysis.

3. Results and discussion

3.1. Gas chromatographic analysis

Gas chromatography with nitrogen phosphorus detection can occasionally cause problems due to changes in sensitivity of the bead in the detector. In this assay, this has not been a problem during daily sample analysis. The bead parameters were optimized daily, and typical runs involved at least 50 plasma samples in addition to standards and quality controls. Bead life often extended to around 1000 samples before the bead needed to be changed.

3.2. Quantification

Peak area measurements were obtained using an HP3350 Laboratory Automation System. The topiramate/internal standard peak area ratios were used to obtain the least squares regression equation. Topiramate concentrations in the human samples and the frozen quality control samples were calculated using measured peak area ratios and the least squares linear regression equation.

3.3. Assay validation

3.3.1. Calibration curves

To establish a calibration curve, a series of

Table 3
Summary of standard curve validation data for topiramate in human urine ($n = 6$)

Concentration (μg ml^{-1})	1.00	2.00	4.00	5.00	10.0	20.0	40.0	50.0
Day 1 Line 1	0.987	2.06	3.99	4.92	10.3	19.9	39.8	49.4
Day 1 Line 2	1.02	1.93	3.97	4.95	10.1	20.3	40.6	49.8
Day 2 Line 1	0.997	2.01	4.04	4.87	10.2	20.5	40.3	48.5
Day 2 Line 2	0.993	2.02	4.00	5.01	10.1	20.1	39.4	49.9
Day 3 Line 1	0.992	2.01	4.04	5.06	10.1	20.2	39.8	48.4
Day 3 Line 2	0.962	2.17	4.00	5.08	10.0	20.1	39.1	48.6
Mean	0.993	2.04	4.01	4.98	10.1	20.2	39.8	49.1
S.D.	0.0197	0.0810	0.0273	0.0822	0.101	0.204	0.537	0.670
% C.V.	1.99	3.98	0.682	1.65	0.994	1.01	1.35	1.36
% Deviation	−747	1.76	0.168	−0.335	1.18	0.821	−0.412	−1.80

standards containing topiramate were prepared inthe range from 0.50 to 50 μg ml^{-1} for plasma and whole blood, and 1.0 to 50 μg ml^{-1} for urine, using 100 μl volumes of the respective human fluid. The samples were extracted as previously described. Duplicate standard curves were run on each of 3 analysis days. The peak area ratios topiramate/internal standard were plotted against concentration. Least squares linear regression analysis gave a calibration equation for topiramate which was used to calculate the concentration of each unknown sample. This assay is shown to be accurate ($\leq$ 2.41% deviation from the theoretical concentrations) and reproducible (C.V. $<$ 4.27%) at each concentration.

The extraction efficiency of the assay procedure was determined by comparing the peak area ratios for topiramate in extracted standards versus those obtained from unextracted standards. The extraction efficiencies of both topiramate and the internal standard were estimated to be $\sim$100% in plasma, using replicates at two concentrations (2 and 40 μg ml^{-1} for topiramate).

The chromatograms shown in Figs. 2–4 indicate that there were no endogenous interfering peaks at the retention times of topiramate or the internal standard, and that the peaks were sharp and well resolved. Typical retention times in human plasma for topiramate were 4.95 and 5.32 min for the internal standard.

A total of six metabolites of topiramate have been identified in humans. The metabolic routes were all relatively minor ($\leq$ 2% of the administered dose) [6]. These metabolites have not caused any chromatographic interference. In the GC-NPD assay, molecules are eluted from the GC column according to their polarity, and due to significant polarity differences, metabolites of topiramate would not be expected to co-elute with topiramate. Two minor hydroxylated metabolites of the isopropylidene groups are both considerably more polar than topiramate, and were shown not to interfere in the GC-NPD assay for topiramate during the development of the assay; retention times later than topiramate were obtained. Minor dihydroxy and trihydroxy metabolites were not extracted or eluted from the GC column under the conditions used for the analysis of topiramate. Minor glucuronide conjugates of the hydroxy metabolites are extremely polar and are weak acids. They would not be extracted under the pH conditions used to prepare the topiramate biological samples. Furthermore, glucuronide conjugates are usually not amenable to direct GC analysis without derivatization due to their lack of volatility and thermal instability, and would, therefore, not be eluted from the GC column during the analysis of topiramate.

3.4. Linearity and precision

3.4.1. Plasma

Standard curve data for topiramate for 3 consecutive days of sample analysis are given in Table

Table 4
Topiramate urine concentrations in frozen quality control samples ($n = 18$)

Theoretical concentration (μg ml^{-1})	3.00	20.0	40.0
Day 1 Line 1	3.07, 2.80, 3.12	19.4, 19.3, 19.1	38.7, 39.1, 40.4
Day 1 Line 2	2.94, 2.93, 2.87	19.2, 19.1, 19.3	38.5, 38.7, 39.4
Day 2 Line 1	2.98, 2.97, 2.94	20.4, 19.9, 21.9	41.6, 40.0, 40.8
Day 2 Line 2	3.08, 2.94, 3.00	19.6, 19.6, 19.3	40.3, 40.7, 40.6
Day 3 Line 1	3.24, 3.30, 3.29	21.4, 21.3, 22.0	42.8, 36.9, 43.2
Day 3 Line 2	3.30, 3.29, 3.34	21.2, 21.0, 21.0	42.0, 43.9, 42.2
Mean calculated conc. (μg ml^{-1})	3.08	20.2	40.5
S.D. from mean	0.173	1.03	1.84
% Acceptance	15.0	15.0	15.0
% C.V.	5.63	5.10	4.55
% Deviation from theoretical	2.57	1.09	1.36

1. The procedure was reproducible from day to day with coefficients of variation (C.V.) of the standards of $< 4.27\%$ for the calculated concentration. Frozen quality control samples at 1.0, 4.0, 10.0 and 40.0 μg ml^{-1} of topiramate were analyzed each day as part of assay validation. The assay is shown to be accurate ($\leq 6.95\%$ deviation from theoretical concentrations) and reproducible (C.V. $< 4.40\%$) at each concentration. These data are presented in Table 2. The frozen quality control samples were assayed for 2 months without a significant change in measured topiramate concentration.

3.4.2. Urine

Standard curve data for topiramate for 3 consecutive days of sample analysis are given in Table 3. The procedure was reproducible from day to day with coefficients of variation (C.V.) of the standards of $< 3.98\%$ for the calculated concentrations. Frozen quality control samples at 3.0, 20.0, and 40.0 μg ml^{-1} of topiramate were analyzed each day as part of assay validation. The assay is shown to be accurate ($\leq 2.57\%$ deviation from theoretical concentration) and reproducible (C.V. $< 5.63\%$) at each control concentration. These data are presented in Table 4.

3.4.3. Whole blood

Standard curve data for topiramate for 3 consecutive days of sample analysis are presented in Table 5. The procedure was reproducible from day to day with coefficients of variation (C.V.) of the standards of $< 4.13\%$ for the calculated concentrations. Frozen quality control samples at 2.0, 12.0, and 25.0 μg ml^{-1} of topiramate were analyzed each day as part of assay validation. The assay is shown to be accurate ($\leq 8.26\%$ deviation from the theoretical concentrations) and reproducible (C.V. $< 5.42\%$) at each concentration. These data are presented in Table 6.

The frozen quality control samples were assayed for 2 months without a significant change in measured topiramate concentrations. Samples may be reinjected up to 24 h after extraction if necessary, with no appreciable change in peak area or calculated concentration. Long-term stability of topiramate in frozen biological samples was confirmed for up to 6 months for plasma and urine. Overall, there were no major stability problems detected for topiramate in biological matrices during long-term storage, freeze/thaw, sample preparation, and analysis.

4. Conclusions

The extraction procedure and the GC-NPD methodology used for the quantitation of topiramate in biological fluids is highly discriminating for topiramate and the internal standard. Chromatograms from the analysis of biological fluids for human, dog, rabbit, and rat plasmas clearly showed that topiramate and the internal standard produced sharp, well-resolved peaks in the chromatograms. Furthermore, the chromatograms

Table 5
Summary of standard curve validation data for topiramate in human blood ($n = 6$)

Concentration (μg ml^{-1})	0.50	1.00	2.00	4.00	5.00	10.0	20.0	40.0	50.0
Day 1 Line 1	0.51	0.94	2.08	4.13	5.04	10.1	20.3	39.4	48.4
Day 1 Line 2	0.49	1.04	1.98	4.01	4.92	10.0	20.5	39.4	49.6
Day 2 Line 1	0.49	1.01	2.04	4.03	4.93	10.2	19.8	39.3	50.1
Day 2 Line 2	0.49	1.04	2.00	4.03	5.07	10.2	20.2	39.1	48.3
Day 3 Line 1	0.50	1.01	1.92	4.09	5.05	10.0	20.5	39.5	49.6
Day 3 Line 2	0.52	0.97	1.92	4.11	5.04	10.3	20.7	40.4	47.3
Mean	0.501	0.999	1.99	4.07	5.01	10.1	20.3	39.5	48.9
S.D.	0.0112	0.0412	0.0650	0.0504	0.0632	0.123	0.338	0.467	1.03
% C.V.	2.23	4.13	3.27	1.24	1.26	1.21	1.66	1.18	2.10
% Deviation	0.158	−0.0713	−0.551	1.66	0.167	1.40	1.71	−1.21	2.26

Table 6
Topiramate whole blood concentrations in frozen quality control samples ($n = 12$)

Theoretical concentration (μg ml^{-1})	2.00	12.0	25.0
Day 1 Line 1	1.83, 1.82	11.5, 11.5	26.8, 26.7
Day 1 Line 2	1.89[a]	12.0, 11.3	25.2, 27.6
Day 2 Line 1	2.08, 2.09	11.2, 11.3	26.9, 25.5
Day 2 Line 2	2.03, 2.05	11.9, 13.4	28.5, 27.2
Day 3 Line 1	1.80, 1.99	12.2, 12.0	27.1, 27.6
Day 3 Line 2	1.97, 2.02	11.8, 12.0	27.7, 28.1
Mean calculated conc. (μg ml^{-1})	1.96	11.9	27.1
S.D. from mean	0.106	0.592	0.964
% Acceptance	15.0	15.0	15.0
N	11	12	12
% C.V.	5.42	4.99	3.56
% Deviation from theoretical	−1.96	−1.16	8.26

[a] Outlier, dropped from regression.

clearly showed the lack of any endogenous or metabolic compounds that would interfere with the quantitation of topiramate concentrations.

This gas chromatography analytical method is currently being used routinely for human clinical samples from pharmacokinetic studies. The assay has been successfully transferred to other analysts and Contract Research Laboratories (CRO) with reliability, reproducibility and robustness.

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