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Automated analysis of a novel anti-epileptic compound, CGP 33 101, and its metabolite, CGP 47 292, in body fluids by high-performance liquid chromatography and liquid–solid extraction

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Abstract

Automated procedures for the determination of CGP 33 101 in plasma and the simultaneous determination of CGP 33 101 and its carboxylic acid metabolite, CGP 47 292, in urine are described. Plasma was diluted with water and urine with a pH 2 buffer prior to extraction. The compounds were automatically extracted on reversed-phase extraction columns and injected onto an HPLC system by the automatic sample preparation with extraction columns (ASPEC) automate. A Supelcosil LC-18 (5 μm) column was used for chromatography. The mobile phase was a mixture of an aqueous solution of potassium dihydrogen phosphate, acetonitrile and methanol for the assay in plasma, and of an aqueous solution of tetrabutylammonium hydrogen sulfate, tripotassium phosphate and phosphoric acid and of acetonitrile for the assay in urine. The compounds were detected at 230 nm. The limit of quantitation was 0.11 μmol/l (25 ng/ml) for the assay of CGP 33 101 in plasma, 11 μmol/l (2.5 μg/ml) for its assay in urine and 21 μmol/l (5 μg/ml) for the assay of CGP 47 292 in urine.

1. Introduction

CGP 33 101 (I) (Fig. 1) is a novel anti-epileptic compound, structurally unrelated to any currently marketed anti-epileptic drug. Following oral administration to man, unchanged I is the major compound in plasma, whereas only traces of the parent compound are recovered in urine (unpublished data). The main metabolite in urine is the carboxylic acid derivative, CGP 47 292 (II) (Fig. 1).

Analytical methods for the determination of unchanged I in plasma and the determination of I and II in urine were required for pharmacokinetic investigations. A method involving liquid–liquid extraction of I from plasma and high-performance liquid chromatography has been published [1]. Brunner and Powell [2] have automated this method by using the Zymate II laboratory automation system. The limit of quantitation was 50 ng/ml.

The present paper describes a method for the determination of I in plasma and a method for the simultaneous determination of I and II in

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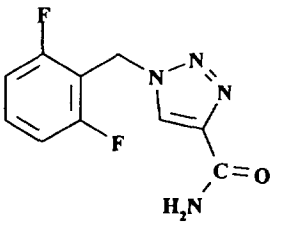
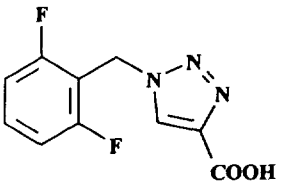
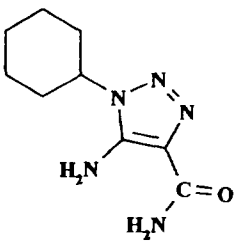


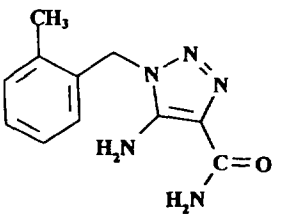
CGP 33 101 (Compound I)



CGP 47 292 (Compound II)



CGP 22 901
(Internal standard, compound III)



CGP 23 901
(Internal standard, compound IV)

Fig. 1. Chemical structure of I, its metabolite II, and the internal standards III and IV.

urine. The method employed liquid–solid extraction via extraction cartridges and was automated by the automatic sample preparation with extraction columns (ASPEC) system. Liquid–solid extraction was applied rather than liquid–liquid extraction because it involves only liquid transfers and is consequently easier to automate.

2. Experimental

2.1. Materials

Compounds I, II and the internal standards CGP 22 901 (III) and CGP 23 901 (IV) were provided by Ciba-Geigy (Basle, Switzerland). The chemical structures of the compounds are shown in Fig. 1. The molecular masses of I and II are 238.2 and 239.2, respectively.

2.2. Reagents

Acetonitrile (HPLC quality), methanol and ethanol (RPE-ACS quality) were from Carlo Erba (Rueil-Malmaison, France). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, tripotassium phosphate trihydrate, ortho-phosphoric acid and Titrisol pH 2 buffer were purchased from Merck (Darmstadt, Germany). Tetrabutylammonium hydrogen sulfate was purchased from Aldrich (Strasbourg, France). Water was purified and deionized using a Milli-Q reagent-grade water system (Millipore, Bedford, MA, USA).

2.3. Apparatus

The chromatographic system consisted of a pump (Model 410, Perkin-Elmer, Norwalk, USA), an ASPEC system (Gilson, Villiers-Le-

Bel, France) and a Model Spectroflow 783 variable-wavelength UV detector (Kratos, Ramsey, NJ, USA) set at 230 nm with a response time of 1 s. A Model C-R4A integrator–recorder (Shimadzu, Kyoto, Japan) was used for data capture.

2.4. Column

A pre-packed column (stainless-steel tube, 15 cm × 4.6 mm I.D.) filled with Supelcosil LC-18, 5 μm (Supelco France, St-Germain-en-Laye, France) was used.

A precolumn (stainless-steel tube, 2 cm × 4.6 mm I.D.) filled with ODS material, 5 μm (Supelco), was placed on top of the column.

A guard-column (stainless-steel tube, 3.3 cm × 4.6 mm I.D.) was placed between the pump and the injector. It was tap-filled in our laboratory with pellicular ODS material, 37–53 μm particle size (Whatman, Clifton, NJ, USA).

2.5. Extraction columns

Bond-Elut extraction columns (100 mg) of 1-ml capacity, C_2 material for the assay in plasma and C_{18} for the assay in urine, were used. They were manufactured by Analytichem International and purchased from Prolabo (Paris, France).

2.6. Calibration solutions

Two solutions of each internal standard were prepared in acetonitrile and in acetonitrile–water (30:70, v/v). Solutions at concentrations of 43 μmol/l (IV) for the assay in plasma and 96 μmol/l (III) for the assay in urine were prepared. Stock calibration solutions at concentrations of 2800 μmol/l of I for the assay in plasma and 420 μmol/l of I and II for the assay in urine were then prepared in the corresponding internal standard solutions in acetonitrile. The calibration solutions were obtained by successive dilutions with the corresponding internal standard solutions prepared in acetonitrile–water.

2.7. Sample handling

Aliquots of 500 μl of plasma, 500 μl of water, 50 μl of internal standard (or calibration) solution, or for the assay in urine, 20 μl of urine, 500 μl of pH 2 Titrisol buffer, 200 μl of internal standard (or calibration) solution, were introduced into a vial and vortex-mixed for a few seconds. Sample handling was then automatically performed by the ASPEC system. The automated sequences are described in Table 1 for the assay in plasma and in Table 2 for the assay in urine.

2.8. Chromatography

Chromatography was performed at room temperature with the mobile phase acetonitrile–methanol–0.02 *M* potassium dihydrogen phosphate (18:6:76, v/v) for the assay in plasma and acetonitrile–0.015 *M* tetrabutylammonium hydrogen sulfate/0.015 *M* tripotassium phosphate/0.003 *M* phosphoric acid (14:86, v/v) for the assay in urine. The flow-rate was 1.2 ml/min for plasma and 1.8 ml/min for urine.

3. Results and discussion

Compound I is a neutral compound poorly soluble in water, acid and alkaline aqueous solutions, but highly soluble in organic solvents. It is well retained on reversed-phase extraction columns at the pH of plasma (pH 7.4). Since it is weakly bound to proteins (the serum protein binding of I is 34%, internal data), plasma samples containing this drug were loaded onto the extraction column following a simple dilution of the sample with water. The extraction yield from plasma was 88% for I (concentration, 2.1 μmol/l) and 91% for IV (concentration, 4.3 μmol/l), as compared with direct injection of a standard solution. However, the acidic metabolite II, ionized at the pH of plasma or urine, was not retained, even on the stronger attracting sorbent C_{18}, following loading in spiked plasma or urine diluted with water. Urine was therefore acidified before loading on a C_{18} extraction

Table 1
Automated procedure for the extraction of I from plasma and the injection of the extract

Step	Liquid dispensed	Dispensing flow-rate (μl/s)	Pressurizing air volume (μl)
Conditioning	· Acetonitrile 1000 μl · Water 1000 μl	100	30
Loading	Diluted plasma 1000 μl	25	400
Washing	· 0.02 *M* K2HPO4 1000 μl · Water 1000 μl	100	400
Elution	Acetonitrile 400 μl	100	400
Dilution of the extract	0.01 *M* KH_2PO_4 2000 μl	800	2500[a]
Injection via a 200-μl loop	Diluted extract 400 μl	25	–

[a] The volume of air was used to mix the extract with water by bubbling.

column in order to increase the affinity of II to the sorbent by ion-suppression. Washing of the extraction column with an acidic solution was not sufficient. Chromatographic interferences were observed. Less interferences were obtained when using a pH 7 washing solvent, but some losses of II occurred. Therefore tetrabutylammonium hydrogen sulfate was added to the pH 7 washing solvent in order to strongly retain II as an ion-pair, whereas I was little influenced by these changes. The extraction yield from urine was 90% for I and 88% for II at concentrations of 84

Table 2
Automated procedure for the extraction of I and II from urine and the injection of the extract

Step	Liquid dispensed	Dispensing flow-rate (μl/s)	Pressurizing air volume (μl)
Conditioning	· Acetonitrile 1000 μl · pH 2 buffer 1000 μl	50	20
Loading	Diluted urine 700 μl	50	100
Washing	Ethanol–salt solution of mobile phase 300 μl (18:82, v/v)	12.5	300
Elution	Ethanol 2 × 150 μl	50	200
Dilution of the extract	Salt solution of mobile phase 3100 μl	200	2500[a]
Injection via a 250-μl loop	Diluted extract 500 μl	25	–

[a] The volume of air was used to mix the extract with water by bubbling.

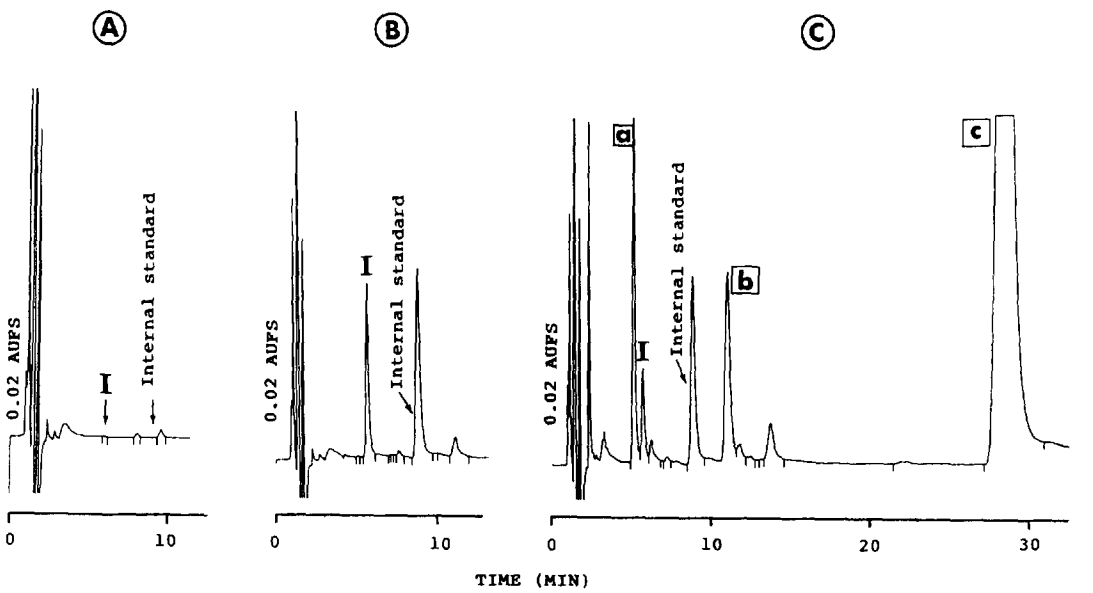


Fig. 2. Chromatograms of extracts of (A) drug-free plasma, (B) spiked plasma containing 4.2 μmol/l of I and 4.3 μmol/l of IV (internal standard) and (C) plasma collected from a patient who took oral doses of carbamazepine and I concurrently. Amount of I found: 2.23 μmol/l. Peaks: a = dihydroxy metabolite of carbamazepine; b = epoxy metabolite of carbamazepine; c = carbamazepine.

μmol/l and 90% for the internal standard (III) at a concentration of 960 μmol/l, as compared with direct injection of a standard solution.

The extract was diluted prior to injection in order to decrease its eluting strength and to avoid band-broadening. A high volume of diluted extract (200–250 μl) was injected and a precolumn was added to protect the analytical column. The precolumn was exchanged every two days for both methods.

As shown in Figs. 2 and 3, I, II and the internal standards were well separated from plasma or urine components. The chromatographic run time was about 12 min and that of sample preparation and injection by the ASPEC system about 10 min for both the assay in plasma and that in urine. A sample was prepared during the chromatography of the previous sample. The diluted plasma and urine samples were found to be stable on the rack of the ASPEC system for at least 15 h at room temperature, thereby allowing high sample throughput.

Calibration graphs were obtained by plotting the peak-height ratio (y) (I or II/internal standard) versus the concentration (x) of either compound in the sample. The equations were

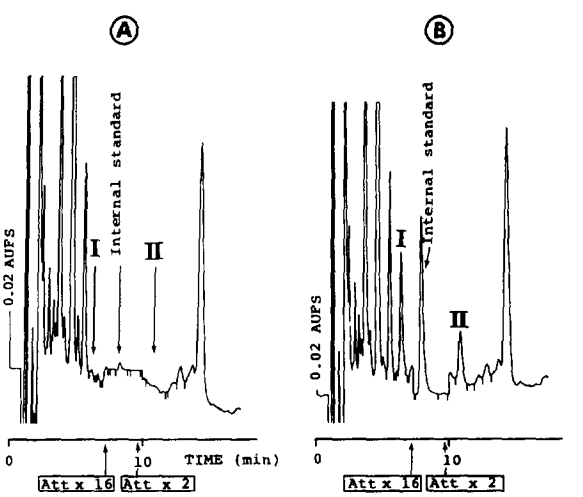


Fig. 3. Chromatograms of extracts of (A) drug-free urine and (B) spiked urine containing 42 μmol/l of I and II and 960 μmol/l of III (internal standard).

calculated by the least-squares method using linear regression with a weighing factor of 1/(concentration)^2 [3]. Typical equations and correlation coefficients (r) were: $y = 14.25x + 0.06$, $r = 0.9991$, for the assay of I in plasma for concentrations ranging from 0.21 to 84 μmol/l when using a 200-μl loop and $y = 12.80x - 0.13$, $r = 0.9995$, when using a 500-μl loop for concentrations ranging from 0.11 to 84 μmol/l; $y = 0.102x - 0.111$, $r = 0.9998$, for the assay of I in urine for concentrations ranging from 11 to 210 μmol/l; $y = 0.040x + 0.055$, $r = 0.9993$, for the assay of II in urine for concentrations ranging from 21 to 840 μmol/l.

The within-day accuracy and precision of the methods were assessed by assaying series of six plasma or urine samples spiked with different concentrations of I or/and II. The results are shown in Table 3. The between-day accuracy and precision were assessed by analysing plasma or urine samples spiked with different concentrations over a 3-week period (Table 4). The limit of quantitation, corresponding to a mean recovery [(found/nominal concentration) · 100] between 85 and 115% and a coefficient of variation lower than 15%, was 0.21 μmol/l (50 ng/ml) for the assay of I in plasma; this could be lowered to 0.11 μmol/l when using a 500-μl loop. The limit of quantitation was 11 μmol/l (2.5 μg/ml) for I and 21 μmol/l (5 μg/ml) for II for the assay in urine.

Compound I was determined in plasma collected from epileptic patients treated with various anti-epileptic drugs and benzodiazepines in addition to I. No interference from phenytoin, valproic acid, carbamazepine, lamotrigine, clobazam, clonazepam, diazepam, nitrazepam and vigabatrin was found. Phenytoin and carbamazepine were eluted with a retention time of about 30 min. The epoxy metabolite and the dihydroxy metabolite of carbamazepine did not interfere as shown in Fig. 2 for a patient who received carbamazepine and I repeatedly. No peaks from the other above mentioned anti-epileptic drugs or their metabolites were observed on the chromatograms. Phenobarbital interfered with the internal standard.

The method for the simultaneous determination of I and its metabolite in urine can be applied for their determination in plasma with minor modifications. However, the limit of quantitation of 1.05 μmol/l is not sufficient for

Table 3
Within-day precision and accuracy of the assays

Fluid	Compound	Nominal concentration (μmol/l)	Mean ($n = 6$) (found/nominal) (%)	C.V. (%)
Plasma	I	0.11[a]	88	15
		0.21	97	4
		0.84	101	3
		4.2	98	1
		21	97	2
		84	95	1
Urine	I	11	100	3
		21	97	2
		42	97	1
		210	99	0.2
	II	21	102	7
		42	102	3
		210	101	0.5
		840	99	0.2

[a] Using a 500-μl loop.

Table 4
Between-day precision and accuracy of the assays

Fluid	Compound	Nominal concentration (μmol/l)	Found/nominal (%)	Mean ± S.D. (%)
Plasma	I	0.11[a]	95, 113	104
		0.21	88, 101, 93, 112	99 ± 10
		0.42	90, 100, 89	93 ± 6
		1.05	102, 105	104
		2.1	104, 102, 100, 102	102 ± 2
		4.2	102, 99, 100, 98, 94	99 ± 3
		11	99, 102, 102, 99	101 ± 2
		21	99, 97, 101, 101, 98	99 ± 2
		42	98, 93, 92	94 ± 3
Urine	I	11	102, 103, 98, 109, 99, 100, 95, 78	98 ± 9
		21	103, 103, 102, 100, 97, 96, 87	98 ± 6
		42	100, 101, 100, 99, 97, 95, 96, 91	97 ± 3
		210	100, 101, 100, 100, 100, 100	100 ± 1
	II	21	94, 122, 106, 112, 110, 113, 114, 84, 84, 94, 95	103 ± 13
		42	94, 117, 109, 105, 110, 97, 99, 92, 99	102 ± 8
		210	98, 103, 101, 101, 97, 102, 101, 101	101 ± 2
		840	98, 99, 96, 101, 101, 91, 100, 91, 98	97 ± 4

[a] Using a 500-μl loop.

the determination of the parent compound for pharmacokinetic studies.

The method for the determination of I in plasma was also automated by using the Zymark BenchMate Workstation. A 500-μl aliquot of plasma (and, for calibration samples, 50 μl of the calibration solution containing only I) was introduced manually into a glass tube which was placed on the rack of the robot until analysis. The internal standard (500 μl of the solution of IV in acetonitrile–water 100-fold diluted with water) was added automatically by the robot. The extraction and injection steps were similar to those described for the ASPEC system, except that the diluted extract was vortex-mixed for 5 s and not stirred by bubbling. The results on accuracy and precision were similar to those obtained by using the ASPEC system.

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