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Isolation of N,N-dialkylated derivatives of valproylglycinamide from dog plasma by active charcoal adsorption and their quantification by high-performance liquid chromatography

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Abstract

A selective assay for quantification of N,N-dimethylvalproylglycinamide (DM-VGD) and N,N-diethylvalproylglycinamide (DE-VGD) in dog plasma utilizing reversed-phase high-performance liquid chromatography and UV detection has been developed. These compounds are derivatives of the potential anticonvulsant drug, valproylglycinamide, which is currently undergoing clinical trials. The method is based on extraction of dog plasma with activated charcoal, separation of the charcoal pellet and extracting it with methanol, evaporation of the solvent and injecting the reconstituted residue onto the column. The active charcoal adsorption method is reliable and reproducible, and it provides a chromatogram free of interfering endogenous plasma compounds. The assay was validated and provided a limit of quantification of 2.3 mmol/l for DE-VGD and 5.3 mmol/l for DM-VGD. Mean recovery of these compounds from plasma averages 75%. This analytical method is suitable for the quantitative determination of DM-VGD and DE-VGD in plasma and it has been applied to a pharmacokinetic study of these compounds in a dog.

Keywords: Valproylglycinamide; N,N-Dimethylvalproylglycinamide; N,N-Diethylvalproylglycinamide; Charcoal, active

1. Introduction

Valproic acid (VPA) is one of the four major existing anti-epileptic drugs. VPA demonstrates a broad spectrum of anti-epileptic activity, but also possesses two serious side effects: hepatotoxicity and teratogenicity [1]. As part of the structure–pharmacodynamic–pharmacokinetic relationship (SPPR) studies conducted in our laboratory, we synthesized valproylglycinamide (VGD, TV-1901, Fig. 1), a conjugate product between VPA and glycinamide [2]. Anti-epileptic animal screening studies found VGD to be a more potent derivative than VPA due to its intrinsic pharmacodynamic properties and better pharmacokinetic profile [2]. In rodents, VGD also possesses a better safety margin than VPA. VGD is

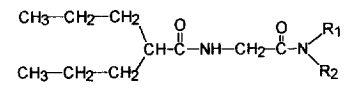

Fig. 1. VGD, $R_1=R_2=H$; DM-VGD, $R_1=R_2=CH_3$; DE-VGD, $R_1=R_2=C_2H_5$.

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not expected to be teratogenic since it does not have a free carboxylic acid [3]. VGD is currently undergoing Phase 1 clinical trials [4].

Our present study was designed to investigate the impact of the N-alkyl substituents of VGD on its pharmacokinetic and anti-epileptic activity. Several N-alkylated VGD derivatives were synthesized and their anticonvulsant activity and neurotoxicity evaluated. N,N-Dimethylvalproylglycinamide (DM-VGD) and N,N- diethylvalproylglycinamide (DE-VGD, Fig. 1) possess improved anti-epileptic activity compared to VPA, and similar activity to that of the parent compound, VGD [5]. In order to assess the relationship between the pharmacodynamics and pharmacokinetics of these drugs, a sensitive and selective analytical method is required.

During the development of an HPLC assay for DM-VGD and DE-VGD in dog plasma using liquid–liquid extraction, satisfactory detection and quantification was not possible due to the co-extraction and co-elution of endogenous lipophilic plasma components. In order to overcome this problem pulverized activated charcoal was used for adsorption of DM-VGD or DE-VGD from dog plasma. In general, silica gel and C_{18} reversed-phase adsorbents are commonly used for liquid–solid extraction procedures.

Activated charcoal is clinically used by humans as an adsorbent in cases of flatulence [6] and drug intoxication [7]. It is also used as a decolorising agent of organic compounds [8] and in air pollution analysis as an adsorbent of organic solvent vapors [9] and volatile aromatic hydrocarbons [10]. Its use as an adsorption agent for quantitative determination of organic compounds in plasma is extremely uncommon [11].

The purpose of this paper is to describe the HPLC procedure used to analyze DM-VGD and DE-VGD in plasma, which utilizes pulverized activated charcoal for their extraction from plasma.

2. Experimental

2.1. Chemicals

Activated charcoal G.R., *tert.*-butyl methyl ether (TBME), thionyl chloride and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Methanol and acetonitrile, HPLC grade, tetrahydrofurane and dichloromethane AR were purchased from Biolab (Jerusalem, Israel). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), glycine, dimethylamine, diethylamine, triethylamine, N-hydroxysuccinimide and sodium hydroxide were purchased from Sigma (St. Louis, MO, USA). VPA was supplied by Teva Pharmaceutical Industries (Jerusalem, Israel).

2.2. Synthesis

Valproyl chloride was prepared by reacting VPA with three equivalents of thionyl chloride in dichloromethane (88% yield). Valproyl glycine was prepared by reacting valproyl chloride in THF with 1.1 equivalents of glycine in the presence of two equivalents of sodium hydroxide (59% yield). DM-VGD was prepared (80% yield) by reacting valproyl glycine with equimolar amounts of EDAC and N-hydroxysuccinimide and three equivalents of dimethylamine and triethylamine. DE-VGD was prepared by the same method as DM-VGD with diethylamine as the nucleophile (46% yield). The chemical structures and purity of all compounds were confirmed by ^{1}H NMR, IR and elementary microanalysis.

2.3. Chromatographic conditions

The HPLC system consisted of an LDC/Milton Roy constametric 3 pump, a manual injection device with a 20-μl loop, a Hewlett-Packard series 1050 detector equipped with a UV lamp and a Hewlett-Packard Model 3395 integrator. The chromatographic separation was achieved on a C_{18} reversed-phase column (LiChrospher, 25 cm, 5 micron, Merck) equipped with a C_{18} reversed-phase guard column (LiChrospher, 1 cm, 5 μm, Merck). The mobile phase composition was an even mixture of acetonitrile and double-distilled water with 0.05% trifluoroacetic acid. Flow-rate was 1.0 ml/min and the eluent was monitored at 220 nm.

2.4. Standard solutions

Standard 1.0 and 0.1 mg/ml stock solutions of DM-VGD and DE-VGD in acetonitrile were kept at

4°C. DM-VGD served as an internal standard of DE-VGD and vice versa. Aliquots of these stock solutions served to prepare spiked plasma samples for calibration curves and assay validations.

2.5. Sample preparation

The following assay was utilized in a study with DM-VGD and DE-VGD in a mongrel dog. Both compounds were dissolved in dimethylsulfoxide and separately injected intravenously to the dog at a dose equivalent to 20 mg/kg of VPA. Blood samples were collected over a 16-h period and transferred into heparinized tubes. The plasma was separated by centrifugation at 3000 *g* for 10 min and stored at −20°C until assayed. Before each assay plasma was allowed to reach room temperature, it was vortexed, centrifuged and the residual clot was removed.

Into tubes containing 0.5 ml of plasma and internal standard, 18–22 mg of active charcoal were added. Tubes contents were mixed by vortexing for 30–45 s and then centrifuged at 3000 *g* for 10 min. The supernatant was discarded and 0.3 ml of distilled water were added to all tubes which were then vortexed for 15 s and centrifuged at 3000 *g* for 5 min. The supernatant was discarded, leaving the charcoal in the tube. Four ml of methanol were then added to each tube and the tubes were vortexed for 45 s and centrifuged at 3000 *g* for 10 min. The methanolic extract was carefully transferred to a new set of tubes and evaporated to dryness under reduced pressure using a vortex evaporator. The dry residue was reconstituted with 100 μl of acetonitrile, of which 40 μl were injected onto the column.

2.6. Calibration curves and assay validation

To construct calibration curves, plasma samples with known concentrations were prepared by addition of DM-VGD and DE-VGD to 0.5 ml blank dog plasma. The samples were processed as described above. Calibration curves were calculated by sum of ordinary least-squares linear regression of the analyte/internal standard peak height ratio vs. the concentration of the analyte. Spiked concentrations of plasma samples ranged from 0.6 to 30 mg/l. The assay was validated on separate occasions using five to eight replicate sets of known concentrations within the aforementioned limits.

3. Results and discussion

As shown in Fig. 2C, under the specified chromatographic conditions DM-VGD elutes at 4.38 min and DE-VGD at 7.20 min. Comparison of ethyl acetate, chloroform, dichloromethane, diethyl ether and TBME extracts of blank dog plasma showed that the latter gave the cleanest background in the HPLC assay (Fig. 2A). Nevertheless, satisfactory separation of DM-VGD and DE-VGD by using TBME extraction was not possible due to the detection of endogenous lipid-soluble compounds. The most troublesome endogenous substance eluted at 4.28 min, unresolved from the peak of DM-VGD, and thus prevented its quantification. The peaks of these endogenous compounds appeared bigger and were accompanied by additional peaks when protein-denaturing agents (acids, sodium hydroxide, acetonitrile and methanol) were added to the plasma prior to TBME extraction.

When activated charcoal used for adsorption was added to blank plasma, separated from the plasma and extracted with methanol, a clean and steady baseline was observed (Fig. 2B). Plasma spiked with DM-VGD and DE-VGD and treated by the same procedure provided a chromatogram free of endogenous substances, while DM-VGD and DE-VGD eluted at 4.38 and 7.20 min, respectively (Fig. 2C).

Calibration curves were linear ($r^2 > 0.997$) within the specified concentration range. Absolute recovery of DM-VGD and DE-VGD is presented in Tables 1 and 2, respectively. These compounds were quantitatively and reproducibly recovered from plasma at the examined concentrations. Results of the assay validation are presented in Tables 3 and 4. Quality control (QC) samples as a means of assay performance during routine analysis resulted in C.V. values of 2.1–4.9% for three different concentrations (5, 10 and 20 mg/l).

It was concluded that both the accuracy and the precision of the charcoal adsorption HPLC method for DM-VGD and DE-VGD in plasma is satisfactory, and it was therefore applied to the pharmacokinetic studies of these compounds in dogs. Dogs are an

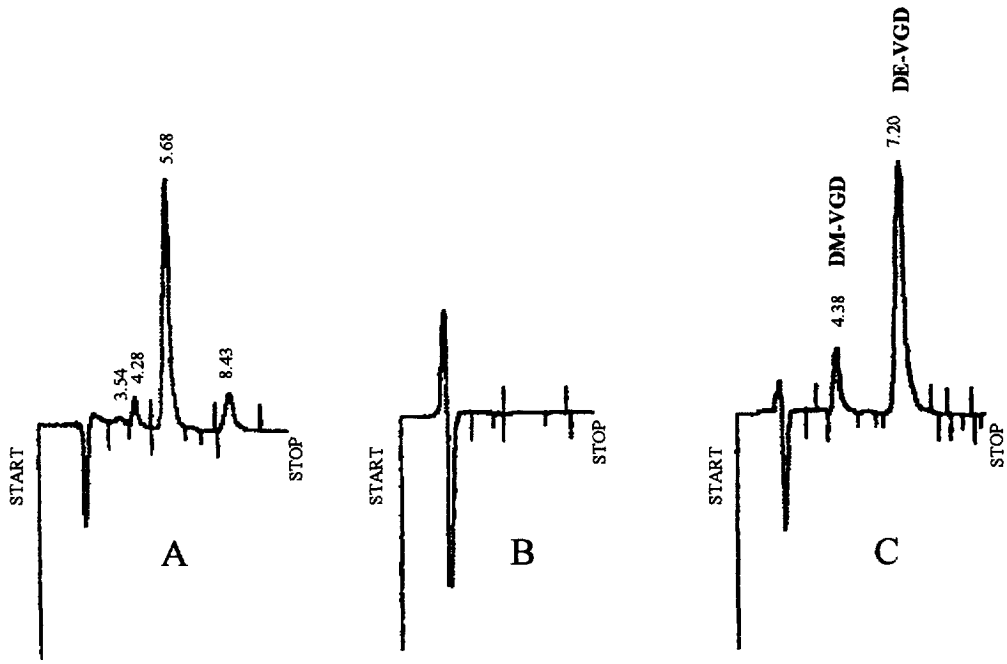

Fig. 2. Typical HPLC chromatograms obtained from dog plasma: (A) *tert.*-Butyl methyl ether extract of blank dog plasma. (B) Blank dog plasma treated with charcoal followed by methanol extraction of the separated charcoal. (C) Blank dog plasma spiked with 10 mg/l DM-VGD and 12 mg/l DE-VGD treated with charcoal and followed by methanol extraction of the separated charcoal.

Table 1
Absolute recovery for DM-VGD (n=3–8 for each concentration)

Concentration (mg/l)	Average (%)	S.D. (%)	C.V. (%)
2	78	4.0	5.1
4	68	4.9	7.3
8	77	6.1	7.9
10	72	4.2	5.8
16	70	7.8	11.1
30	85	2.5	2.9
Overall	75	6.9	9.3

Table 2
Absolute recovery for DE-VGD (n=3–5 for each concentration)

Concentration (mg/l)	Average (%)	S.D. (%)	C.V. (%)
2	89	5.9	6.6
4	63	5.9	9.4
8	69	8.6	12.5
16	71	12.0	17.1
30	69	2.7	3.8
Overall	72	7.0	9.9

S.D., standard deviation; C.V., coefficient of variation.

excellent animal model for cross-over pharmacokinetic studies which can be extrapolated to humans. A representative graph of pharmacokinetic data obtained from the dog is presented in Fig. 3.

The very low cost of active charcoal, and the relative ease and safety of its use, make it a good alternative, in certain cases, to silica gel and C_{18} reversed-phase columns commonly used in solid–liquid extraction procedures. Recovery of the compounds adsorbed to the charcoal beads can be best achieved using water-miscible solvents such as methanol, ethanol, acetonitrile, etc. Organic solvents which are not water-soluble cannot suspend the charcoal pellet efficiently and therefore do not extract the adsorbed compound quantitatively as do the former solvents.

In summary, the analytical method described here is reproducible, sensitive and accurate and is suitable for the pharmacokinetic analysis of DM-VGD, DE-VGD and might also be applied to other N-alkylated VGD derivatives.

Table 3
Assay validation for DM-VGD ($n=5$)

Spiked concentration (mg/l)	Measured concentration (mg/l)	S.D. (mg/l)	C.V. (%)	R.E. (%)
1.2	1.24	0.05	4.13	3.56
2.4	2.30	0.04	1.85	−4.36
5	4.58	0.15	3.36	−8.35
8	7.18	0.14	1.90	−10.20
10	9.45	0.57	6.08	−5.47
14	13.92	0.67	4.81	−0.54
20	19.65	1.28	6.53	−1.75
30	32.53	1.62	4.98	8.44

S.D., standard deviation; C.V., coefficient of variation (precision); R.E., relative error (accuracy).

Table 4
Assay validation for DE-VGD ($n=8$)

Spiked concentration (mg/l)	Measured concentration (mg/l)	S.D. (mg/l)	C.V. (%)	R.E. (%)
0.6	0.52	0.05	9.90	−13.52
1.2	1.09	0.12	11.25	−9.25
2.4	2.26	0.33	14.77	−5.68
5	5.14	0.26	5.11	2.80
10	10.19	0.98	9.65	1.92
20	21.34	2.23	10.44	6.72
30	31.91	3.63	11.36	6.36

S.D., standard deviation; C.V., coefficient of variation (precision); R.E., relative error (accuracy).

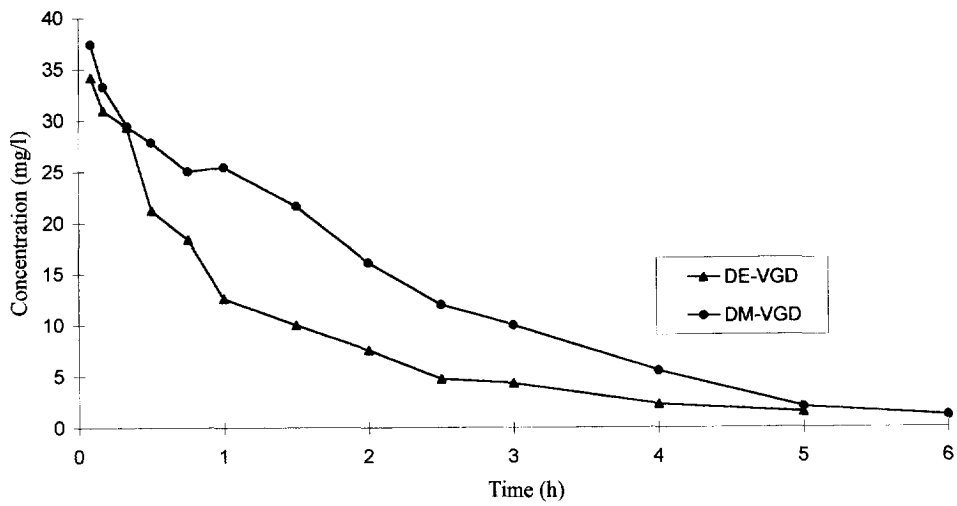

Fig. 3. Plasma concentrations of DM-VGD and DE-VGD obtained after intravenous administration (728 mg of DM-VGD and 781 mg of DE-VGD) to dog #2652.

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