ELSEVIER

Journal of Chromatography B, 761 (2001) 255–259

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

High-performance liquid chromatographic determination of diazepam in plasma of children with severe malaria

S.N. Muchohi[a,*], B.R. Ogutu[b,c,d], C.R.J.C. Newton[d,e], G.O. Kokwaro[a,b]

[a]*Kenya Medical Research Institute (KEMRI)/Wellcome Trust Collaborative Research Programme, P.O. Box 43640, Nairobi, Kenya*

[b]*Department of Pharmaceutics and Pharmacy Practice, University of Nairobi, P.O. Box 19676, Nairobi, Kenya*

[c]*KEMRI-Centre for Clinical Research, P.O. Box 54840, Nairobi, Kenya*

[d]*KEMRI/Wellcome Trust Collaborative Research Programme, Centre for Geographic Medicine Research-Coast, P.O. Box 230, Kilifi, Kenya*

[e]*Neurosciences Unit, Institute of Child Health, University of London, London, UK*

Received 26 January 2001; received in revised form 21 May 2001; accepted 30 May 2001

Abstract

A sensitive, selective and reproducible reversed-phase HPLC method with ultraviolet detection was developed for the quantification of diazepam in small plasma samples from children with severe malaria. The method involves plasma deproteinization with acetonitrile, followed by liquid–liquid extraction with ethyl acetate–*n*-hexane. Diazepam was eluted at ambient temperatures from a reversed-phase C_{18} column with an acidic (pH 3.5) aqueous mobile phase (10 m*M* KH_2PO_4–acetonitrile, 69:31, v/v). Calibration curves in spiked plasma were linear from 10 to 200 ng ($r^2 \geq 0.99$). The limit of detection was 5.0 ng/ml, and relative recoveries at 25 and 180 ng were >87%. Intra- and inter-assay relative standard deviations were <15%. There was no interference from drugs commonly administered to children with severe malaria (phenobarbitone, phenytoin, chloroquine, quinine, sulfadoxine, pyrimethamine, halofantrine, cycloguanil, chlorcycloguanil, acetaminophen and salicylate). This method has been used for monitoring plasma diazepam concentrations in children with seizures associated with severe malaria. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Diazepam

1. Introduction

Severe malaria is a major cause of paediatric admission in the Tropics [1,2], and is associated with an estimated case-fatality rate of 10–30% [3–6]. Seizures are a clinical feature of severe malaria in children [1,6,7], and associated with an increase in mortality [7] and neurological sequalae [8]. Diazepam (DZ) is the drug of choice for control of acute seizures. It is cheap, widely available in resource poor countries and effective when given by the intravenous or rectal routes. Studies of the clinical pharmacology of DZ may require determination of concentrations of DZ in biological fluids. For studies in children with severe malaria, the ideal analytical method for DZ should be sensitive enough to allow use of small quantities of biological fluid (since these children often have severe anaemia), and selective to avoid interference from concurrently administered drugs (such as other anticonvulsants,

*Corresponding author. Fax: +254-2-711-673.

E-mail address: Sndirangu@wtnairobi.mimcom.net (S.N. Muchohi).

0378-4347/01/$ – see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(01)00284-5

antimalarial drugs, antibiotics and analgesic/antipyretics).

Several analytical techniques for quantifying DZ in biological fluids have been reported, including gas chromatography (GC) with nitrogen-sensitive [9,10] or electron-capture detection [11] and high-performance liquid chromatographic (HPLC) methods [12–23]. Most of the methods are limited by large sample volumes (1.0–2.0 ml) [13–15,18,20,22], laborious extraction procedures [17,18,21], and long retention times [19–21,23]. For studies in children with malaria, some of these methods [15,16] may not be selective enough to allow quantitation of DZ in presence of antimalarial drugs.

We report a simple, sensitive and selective HPLC method that requires only 100 μl plasma for the determination of DZ. We have used this method to monitor plasma DZ concentrations in a group of children with severe malaria who had been administered DZ to control associated seizures.

2. Experimental

2.1. Chemicals and reagents

Reference DZ, clonazepam (CLZ), quinine, chloroquine, sulfadoxine, pyrimethamine, chloramphenicol, acetaminophen, and salicylic acid were obtained from the Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol, ethyl acetate, *n*-hexane, dichlorodimethylsilane (used to silanize glassware) and orthophosporic acid were purchased from BDH (Poole, UK). Boric acid, potassium chloride, potassium dihydrogenorthophosphate, sodium carbonate (all AnalaR grade) were obtained from BDH.

2.2. Extraction procedure

To 100 μl aliquots of blank plasma spiked with DZ or patient plasma in 15-ml PTFE-lined screw-capped glass centrifuge tubes, was added 10 μl methanol containing CLZ (the internal standard, 100 ng) followed by 300 μl acetonitrile (protein precipitant). The mixture was vortex-mixed (30 s) and then centrifuged (1600 *g*, 5 min). The clear supernatant was transferred to a clean glass centrifuge tube containing borate buffer (0.5 ml; pH 9.0), and extracted with ethyl acetate–*n*-hexane (30:70, v/v; 5 ml) mixture by end-over-end mixing (33 rpm; 30 min) on a Stuart SB1 blood tube rotator (Stuart Scientific, Bedfordshire, UK). Following centrifugation (1600 *g*, 5 min) and separation, the upper organic phase was evaporated to dryness in a water bath (37°C) under a gentle stream of white spot nitrogen gas. Samples were reconstituted in mobile phase (100 μl) and 50-μl aliquots were injected onto the column.

2.3. Preparation of standard curves

Stock solutions (1 mg/ml) of DZ and CLZ (internal standard) were prepared by dissolving 10 mg of each compound in 10 ml of methanol. Working solutions (100, 10, 1 μg/ml) were prepared by appropriate serial dilution of the stock solutions with methanol. Known amounts (10–200 ng) of DZ and a fixed amount (100 ng) of CLZ were added (in duplicate) to blank human plasma, and extracted and analysed by the described procedures. Peak height ratios (drug/internal standard) were plotted as a function of the known amounts of DZ, and the results analysed by least-squares linear regression.

2.4. Chromatography

Chromatography was performed under isocratic conditions. An isochrom delivery system (Isochrom LC; Spectra Physics, San Jose, CA, USA) fitted with a Model 7125 Rheodyne (Cotati, CA, USA) valve injector (50-μl loop) was used. A stainless steel C_{18} column (Ultrasphere ODS, 15 cm×4.6 mm I.D., 5 μm particle diameter; Beckman Instruments, Milford, MA, USA) preceded by a guard column (LiChrospher 100 RP-18 endcapped, 5 μm, 4 cm× 4.6 mm I.D.; Merck, Darmstadt, Germany) was operated at ambient temperature. The mobile phase comprised 10 m*M* KH_2PO_4–acetonitrile (69:31, v/v), and adjusted to pH 3.5 with orthophosphoric acid. The flow-rate was 2.0 ml/min, generating an operating back-pressure of approximately 120 bar. The column effluent was monitored with a variable wavelength UV–Vis detector (Model Spectra 100, Spectra Physics) set at 228 nm. Chromatographic peaks were recorded on a flatbed chart recorder (Servogor 120, BBC Goerz Metrawatt, Belmont Instruments, Glasgow, UK).

2.5. Recovery and reproducibility

The analytical recovery of the extraction procedure for DZ was assessed by spiking 100-μl aliquots of drug-free plasma with 25 and 180 ng ($n=10$ for each level) of DZ and 100 ng of internal standard. The samples were processed as described in the extraction procedure above. In another set of tubes, equivalent amounts of DZ together with 100 ng of internal standard were added directly into extraction solvent (5 ml), followed by evaporation of the solvent in a water bath under a gentle stream of nitrogen. The residue was reconstituted in mobile phase and injected into the chromatograph as described above. The recovery was assessed by comparison of the peak height ratios of DZ to internal standard for the extracted spiked plasma samples, with those from equivalent amounts of DZ spiked directly into the organic phase.

Reproducibility of the analytical procedure was assessed by determination of intra- and inter-day relative standard deviations (RSDs) for amounts of DZ in spiked plasma (100 μl). Intra-assay (within-day) variability was assessed by analysing six replicates of plasma samples spiked with DZ at three different amounts (25, 80 and 180 ng). Inter-assay (day-to-day) variability was assessed by analysing duplicates for each amount over 5 consecutive days.

2.6. Selectivity

Various anti-malarial drugs (chloroquine, quinine, sulfadoxine, pyrimethamine, halofantrine, proguanil, cycloguanil, chlorproguanil), other anticonvulsants (phenobarbitone, phenytoin, fosphenytoin, paraldehyde), analgesics (acetaminophen, salicylate) and antibiotics (chloramphenicol) commonly used in management of severe malaria and associated complications were evaluated for interference with the assay for DZ. Drug-free plasma was spiked with therapeutic concentrations of these drugs followed by extraction and analysis as described above.

2.7. Clinical study

Blood samples (0.4 ml predose and at 5, 10, 20, 30, 40, 60 min, and 2, 4, 6, 8, 12, 24 and 48 h) were obtained from children who had been enrolled into a separate study on the clinical pharmacology of DZ in children with seizures associated with severe malaria [24]. The children had been administered DZ either intravenously (0.3 mg/kg as a slow infusion over 1 min) or rectally (0.5 mg/kg of the injection solution). Plasma was obtained by centrifugation (1600 *g*, 5 min), and aliquots (100 μl) processed and assayed for DZ by the procedures described. Concentration–time profiles for two of the patients (one from the intravenous DZ and one from the rectal DZ group) are reported.

3. Results and discussion

3.1. Chromatography

DZ and CLZ (internal standard) were resolved to baseline throughout the calibration ranges of 10–200 ng, with retention times of 4.5 and 11.0 min for CLZ and DZ, respectively (Fig. 1). There was no interference from endogenous compounds or any of the drugs screened. The limit of detection was 5.0 ng/ml, which at 0.002 absorbance units full-scale (aufs) consistently produced a peak at least four times the background noise.

3.2. Recovery, calibration curves and reproducibility

The percentage recoveries (mean±SD) at 25 and 180 ng of DZ were 87.1±4.7 ($n=9$) and 96.9±1.91 ($n=8$), respectively. The calibration curves in spiked plasma were linear within the range 10–200 ng ($r^2 \geq 0.99$).

The intra-assay RSDs at 25, 80 and 180 ng of DZ were 6.4, 9.0 and 5.1%, respectively ($n=6$ in all cases). The inter-assay RSDs at the above concentrations were 5.6, 14.1 ($n=5$ for both) and 11.2% ($n=4$), respectively.

3.3. Clinical study

This assay method was successfully used to quantitatively measure the concentrations of DZ in plasma samples obtained from paediatric patients who were administered diazepam either intravenously (0.3 mg/kg) or rectally (0.5 mg/kg). Fig. 2 shows concentration–time profiles from two of the patients. *N*-desmethyldiazepam (*N*-DMDZ), the main pharma-

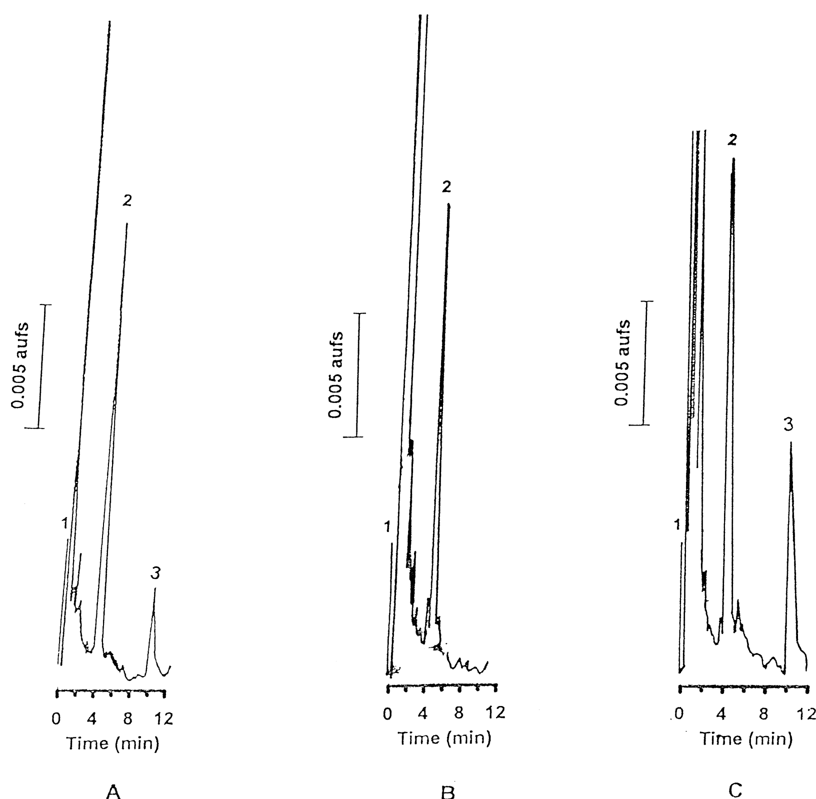

Fig. 1. Chromatograms of 100-μl extracts of: (A) plasma spiked with CLZ (internal standard; 100 ng) and DZ (20 ng); (B) predose plasma from a paediatric patient spiked with CLZ (100 ng); and (C) plasma from the same patient obtained 5 min following intravenous administration of DZ (0.3 mg/kg) and spiked with CLZ (100 ng). DZ concentration was 583 ng/ml; *N*-DMDZ was not detected. Peaks: 1=injection; 2=CLZ; 3=DZ.

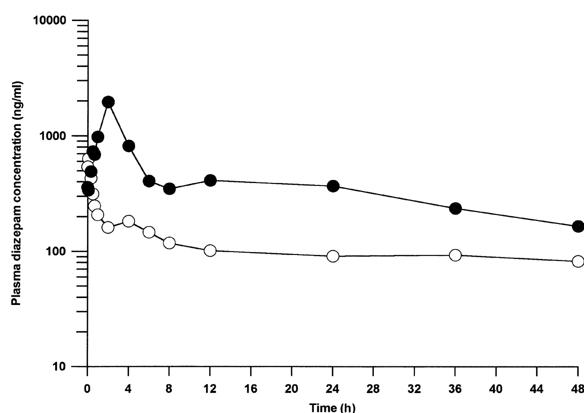

Fig. 2. Plasma diazepam concentration versus time profiles following the administration of DZ either intravenously (0.3 mg/kg as a slow infusion over 1 min; open circles) or rectally (0.5 mg/kg of the injection solution; closed circles) to two paediatric patients.

cologically active metabolite of DZ, was undetectable in all patients studied.

A previous study [25] using a GC method was able to detect *N*-DMDZ levels in 100 μl of plasma from two children administered anticonvulsant doses of DZ. However, the same children had also received DZ intramuscularly and rectally, and it is possible that *N*-DMDZ had accumulated to detectable levels. The anticonvulsant effect of *N*-DMDZ is thought to be lower than that of DZ [25,26], and probably negligible following single dose administration. Thus, although the present method is not sensitive enough to detect *N*-DMDZ in 100 μl of plasma following administration of a single 0.3 mg/kg intravenous dose or 0.5 mg/kg rectal dose of DZ, the method has the practical advantages of being rapid, selective and cheap. It is suitable for determination

of DZ concentrations, which is the more relevant parameter following administration for acute seizure control.

In conclusion, we have described a HPLC method for the determination of DZ that is specific, simple and rapid. The assay requires a small sample volume (100 μl) which is important in clinical studies in young children. The method has been applied in a study involving the monitoring of DZ concentrations in children with malaria, thus validating its clinical utility in this group.

Acknowledgements

We thank the Director of the Kenya Medical Research Institute (KEMRI) for permission to publish this work. G.O.K. acknowledges financial support from the Wellcome Trust (grant No. 057978/Z/99/Z) and the WHO (MIM Research Capability Strengthening in Africa, grant No. 980074). C.R.J.C.N. holds a Wellcome Trust Career post in Tropical Medicine (grant No. 050533), while B.R.O. is a graduate trainee in Clinical Pharmacology funded by the WHO.

References

[1] K. Marsh, D. Forster, C. Waruiru, I. Mwangi, M. Winstanley, V. Marsh, C. Newton, P. Winstanley, P. Warn, N. Peshu, G. Pasvol, R. Snow, New Engl. J. Med. 332 (1995) 1399.
[2] D.R. Brewster, B.M. Greenwood, Ann. Trop. Paediatr. 13 (1993) 133.
[3] D.A. Warrell, M.E. Molyneux, P.F. Beales, Trans. R. Soc. Trop. Med. Hyg. 84 (Suppl. 2) (1990) 1.
[4] M.E. Molyneux, T.E. Taylor, J.J. Wirima, A. Borgstein, Q. J. Med. 71 (1989) 441.
[5] D. Sturchler, Parasitol. Today 5 (1990) 12.
[6] C.R.J.C. Newton, S. Krishna, Pharmacol. Ther. 79 (1998) 1.
[7] J. Crawley, S. Smith, F. Kirkham, P. Muthinji, C. Waruiru, K. Marsh, Q. J. Med. 89 (1996) 591.
[8] D.R. Brewster, D. Kwiatkowski, N.J. White, Lancet 336 (1990) 1030.
[9] M. Krogh, H. Grefslie, K.E. Rasmussen, J. Chromatogr. B 689 (1997) 357.
[10] C. Lora-Tamayo, M.A. Rams, J.M. Chacon, J. Chromatogr. 374 (1986) 73.
[11] H. Gjerde, E. Dahlin, A.S. Christophersen, J. Pharm. Biomed. Anal. 10 (1992) 317.
[12] A.K. Dhar, H. Kutt, Clin. Chem. 25 (1979) 137.
[13] Y. Caraco, T. Tateishi, A.J.J. Wood, Clin. Pharmacol. Ther. 58 (1995) 62.
[14] T. Ishizaki, K. Chiba, K. Manabe, E. Koyoma, M. Hayashi, S. Yasuda, Y. Horai, Y. Tomono, C. Yamato, T. Toyoki, Clin. Pharmacol. Ther. 58 (1995) 155.
[15] A. Tracqui, P. Kintz, P. Mangin, J. Forensic Sci. 40 (1995) 254.
[16] H. Vendenberghe, J.C. MacDonald, Ther. Drug. Monit. 17 (1995) 393.
[17] H. Iwase, K. Gondo, T. Koike, I. Ono, J. Chromatogr. 655 (1994) 73.
[18] J.H. Nichols, J.R. Charlson, G.M. Lawson, Clin. Chem. 40 (1994) 1312.
[19] C. Lacroix, F. Wojciechowski, P. Danger, J. Chromatogr. 617 (1993) 285.
[20] M. Estadieu, A. Durand, A. Viala, P.P. Rop, M. Fornaris, J. Quicke, J. Anal. Toxicol. 13 (1989) 89.
[21] M.J. Koenigbauer, M.A. Curtis, J. Chromatogr. 427 (1988) 277.
[22] K. Tada, T. Moroji, R. Sekiguchi, H. Motomura, T. Noguchi, Clin. Chem. 31 (1985) 1712.
[23] P.M. Kabra, B.E. Stafford, L.J. Marton, J. Anal. Toxicol. 5 (1981) 177.
[24] B.R. Ogutu, C.R.J.C. Newton, J. Crawley, S.N. Muchohi, G.O. Otieno, G. Edwards, G.O. Kokwaro, Br. J. Clin. Pharmacol. (2001) submitted for publication.
[25] S. Agurell, A. Berlin, H. Ferngren, B. Hellstrom, Epilepsia 16 (1975) 277.
[26] F.U. Knudsen, Acta Paediatr. Scand. 66 (1977) 563.