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Determination of 5-chloro-7-iodo-8-quinolinol (clioquinol) in plasma and tissues of hamsters by high-performance liquid chromatography and electrochemical detection

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Abstract

This paper describes a method of determining clioquinol levels in hamster plasma and tissue by means of HPLC and electrochemical detection. Clioquinol was separated on a Nucleosil C18 300 mm × 3.9 mm i.d. 7 μm column at 1 ml/min using a phosphate/citrate buffer 0.1 M (400 ml) with 600 ml of a methanol:acetonitrile (1:1, v/v) mobile phase. The retention times of clioquinol and the IS were, respectively, 11.6 and 8.1 min; the quantitation limit (CV > 8%) was 5 ng/ml in plasma and 10 ng/ml in tissues. The intra- and inter-assay accuracies of the method were more than 95%, with coefficients of variation between 3.0 and 7.7%, and plasma and tissue recovery rates of 72–77%. There was a linear response to clioquinol 5–2000 ng/ml in plasma, and 10–1000 ng/g in tissues. The method is highly sensitive and selective, makes it possible to study the pharmacokinetics of plasma clioquinol after oral administration and the distribution of clioquinol in tissues, and could be used to monitor plasma clioquinol levels in humans.

Keywords: Clioquinol; Hamsters; HPLC; Electrochemical detection

1. Introduction

Clioquinol (5-chloro-7-iodo-8-quinolinol) acts as a zinc and copper chelator. Metal chelation is a potential therapeutic strategy for Alzheimer's disease because the interaction of zinc and copper is involved in the deposition and stabilisation of amyloid plaques, and chelating agents can dissolve the amyloid deposits by preventing metal-A-beta interactions [1–3]. As Alzheimer's disease and prion disease are CNS degenerative disorders characterised by amyloid deposits, it is conceivable that some drugs may be active in preventing both.

Transmissible spongiform encephalopathies (TSE) form a group of progressive, fatal neurodegenerative diseases affecting the central nervous system of humans (kuru, Creutzfeldt-Jacob disease) and animals (scrapie, bovine spongiform encephalopathy) [4–6]. It is believed [7] that the causative agents are proteinaceous infectious particles ("prions") completely devoid of any nucleic acids that represent the altered counterpart of a cell protein, and are resistant to proteolytic digestion, high temperatures, denaturating agents and the disinfectants usually used for sterilisation. The pathological protein (PrP^{sc}) is the protease-resistant isoform of a GPI-anchored cell transmembrane molecule (PrP^{c}) that is mainly expressed in CNS neurons, but also in many other cell types. As it is the main component of amyloid deposits, and the cause of neurodegenerative CNS lesions, PrP^{sc} is the primary target for therapeutic strategies [8,9].

The hamster model is particularly suitable for TSE studies because the period required for the development of experimental scrapie is shorter than in mouse; when hamsters are intracerebrally infected by the 263 K prion strain, the incubation period lasts 2 months and death occurs after about 1 month [10,11]. Preliminary results indicate that clioquinol may improve

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cognitive symptoms and prolong the survival of infected animals [12].

After oral administration in rodents (mice and rats, but not hamsters), clioquinol is extensively metabolised to glucuronate and sulfate metabolites [13–18], but these animal and human studies made use of relatively insensitive and non-specific HPLC methods with UV detection, and thus required complex extraction procedures in order to determine tissue clioquinol levels. An even more complex GC method with electron-capture detection after acetylation has been developed by Jack and Riess [19], which also used solvent extraction with a sensitivity of 50 ng/ml. Finally, a highly sensitive GC–MS method has been developed that uses benzene extraction and the conversion of clioquinol into pentafluorobenzyl ether [20].

As studying the pharmacokinetics of clioquinol and its tissue distribution may be relevant to understanding its targets and its mechanism of inhibiting prion infection, we have developed a simple, sensitive and specific method of determining clioquinol levels in plasma and tissues by means of HPLC and electrochemical detection.

2. Experimental methods

2.1. Chemicals and reagents

The 5-chloro-7-iodo-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline, phenolphthalein glucuronic acid and β-glucuronidase (25,000 units/0.4 ml) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and the analytical reagents from Merck (Darmstadt). The aqueous solutions were in reagent-grade water obtained using a Milli-Q System (Millipore, Bedford, MA, USA). Stock solutions (0.5 mg/ml in methanol) were prepared every month and stored at 4 °C. In these conditions solutions have found stable for more than 1 month. Standard working solutions were prepared every day immediately before use in distilled water.

2.2. Biological samples

The plasma, and the brain, spleen and liver tissues were obtained from Syrian hamsters treated orally with clioquinol 50 mg/kg. Sixty minutes after administration, the animals were anesthetised with diethyl ether, their blood was drawn by heart puncture and centrifuged, and the plasma was stored at −25 °C until analysis. After the animals were sacrificed, the tissues were removed, washed with physiological solution, wiped with clean paper, weighed, and frozen at −25 °C.

At the beginning of the study, three series of standard plasma and tissue samples from drug-free animals were prepared as described below, and spiked with low, medium and high concentrations of clioquinol (10, 100 and 2000 ng/ml in plasma; 20, 100 and 500 ng/ml in tissues: quality control samples), and frozen at −25 °C. One sample of each known concentration was analysed at each run of the unknown samples. The samples were analysed within 2 weeks.

2.3. Sample preparation

2.3.1. Plasma

A volume of 0.2 ml of plasma was deproteinised with an equal volume of 0.6N perchloric acid containing sodium disulfite 1 mg/ml, disodium ethylendiaminotetracetate (EDTA) 0.5 mg/ml and 40 ng of 5,7-dichloro-8-hydroxyquinoline as internal standard (IS); methanol 0.2 ml was added and mixed by vortexing. The antioxidant disulfite was added to protect clioquinol from possible degradation due to the presence of perchloric acid; EDTA was added to prevent the formation of complexes that may reduce recovery and be induced by clioquinol (a potent chelator of many ions); and methanol was added to improve the solubility of clioquinol in the extraction medium. The lowest possible methanol concentration was used in order to prevent the extraction of other interfering compounds.

The proteins were precipitated by centrifugation at 13,000 × g × 15 min at 4 °C; 50 μl of the clear acid supernatant were suitable for use in the chromatographic system.

Standard plasma samples were prepared by spiking 0.2 ml of drug-free plasma with known amounts of clioquinol (5–2000 ng/ml) and 40 ng of IS. The samples were analysed as described above.

2.3.2. Tissue

Brain, spleen and liver (about 450 mg of brain, 150 mg of spleen, 200 mg of liver) were homogenised in an Ultra-Turrax apparatus with 1 ml of 0.6N perchloric acid and 120 ng of internal standard; methanol 0.5 ml was added and the mixture was re-homogenised. After centrifugation as above, 50 μl of the supernatant were injected into the chromatographic system.

Standard tissue samples obtained from drug-free animals were prepared by spiking before homogenisation 1 ml of 0.6N perchloric acid with known amounts of clioquinol (10–1000 ng/ml) and 120 ng of IS. The samples were analysed as described above.

Calibration curves were calculated by analysing the linear regression of the ratios of the peak clioquinol and IS area against the clioquinol concentrations in the standard samples, and the curves were used to calculate the concentrations of clioquinol in the unknown samples.

2.4. Clioquinol glucuronate and sulfate

Clioquinol glucuronate was determined in plasma according to the method of Chen et al. [21] with modifications: after enzymatic hydrolysis: 400 μl of distilled water, 100 μl of 1 M acetate buffer, pH 5, and β-glucuronidase at a final concentration of 200 units/ml were added to a sample of 500 μl of plasma; phenolphthalein glucuronide was used as a standard to check the efficiency of the deconjugation reaction. The samples were incubated at 37 °C for 2 h, and the unconjugated clioquinol and IS were extracted and determined as previously described. To check the deconjugation reaction, the samples were reinjected under different chromatographic conditions (see below) in order to obtain adequate separation and unconjugated phenolphthalein

concentrations determined in comparison to an external standard.

Clioquinol sulfate was determined after acid hydrolysis according to the method of Chen et al. [21]: 0.2 ml of plasma were deproteinised with an equal volume of 0.6N perchloric acid containing sodium disulfite 1 mg/ml, disodium ethylendiaminotetracetate 0.5 mg/ml and 40 ng of the internal standard; methanol 0.2 ml was added and mixed by vortexing. After centrifuging at 13,000 × *g* × 15 min at 4 °C, a 200 μl aliquot of the supernatant was heated at 95° for 12 min, and then 50 μl were injected into the chromatograph.

2.5. Liquid chromatography

HPLC analysis was carried out using a Shimadzu system (Kyoto, Japan) consisting of an LC-6A HPLC pump, an SIL-9A refrigerated autosampler, an SCL-6A Controller, and a CR4-A Integrator. The detector was an ESA amperometric Coulochem dual potententiostat 5100 (Bedford, USA). Clioquinol was separated from the IS and the interfering plasma and tissue peaks using a 300 mm × 3.9 mm i.d., 7 μm Nucleosil C18 column.

The analytes were detected using a dual electrode analytical cell (Model 5011A), with the first electrode (E1) set at +300 mV and the second (E2) at +700 mV. A guard cell (Model 5020) between the pump and autosampler was set at +750 mV. All of the potentials were chosen on the basis of voltammograms.

The mobile phase consisted of a phosphate/citrate buffer 0.1 M (400 ml) with the addition of 600 ml of methanol: acetonitrile (1:1, v/v), and was delivered at a flow rate of 1.0 ml/min. Phenolphtalein concentrations were determined using a different mobile phase with a decreased acenotrile/methanol component (600 ml of phosphate/citrate buffer 0.1 M and 400 ml of methanol:acetonitrile [1:1, v/v]): under these conditions, the phenolphthalein, IS and clioquinol retention times were, respectively, 7.1, 29.4 and 56.3 min.

All of the analyses were performed at 22 °C (LC 23 temperature controller, BAS).

2.6. Accuracy and precision

Accuracy and precision were evaluated using the values obtained following same-day replicated analyses of five standard samples ($n = 10$) for intra-day accuracy and precision, and daily analyses of three standard quality control standard samples at low, medium and high concentrations (10, 100 and 2000 ng/ml in plasma; 20, 100 and 500 ng/ml in tissue) for inter-day accuracy and precision. Accuracy was calculated as the percentage of the known concentrations actually measured; precision was determined as the coefficient of variation (CV): i.e. the ratio between the SD and the mean measured concentration.

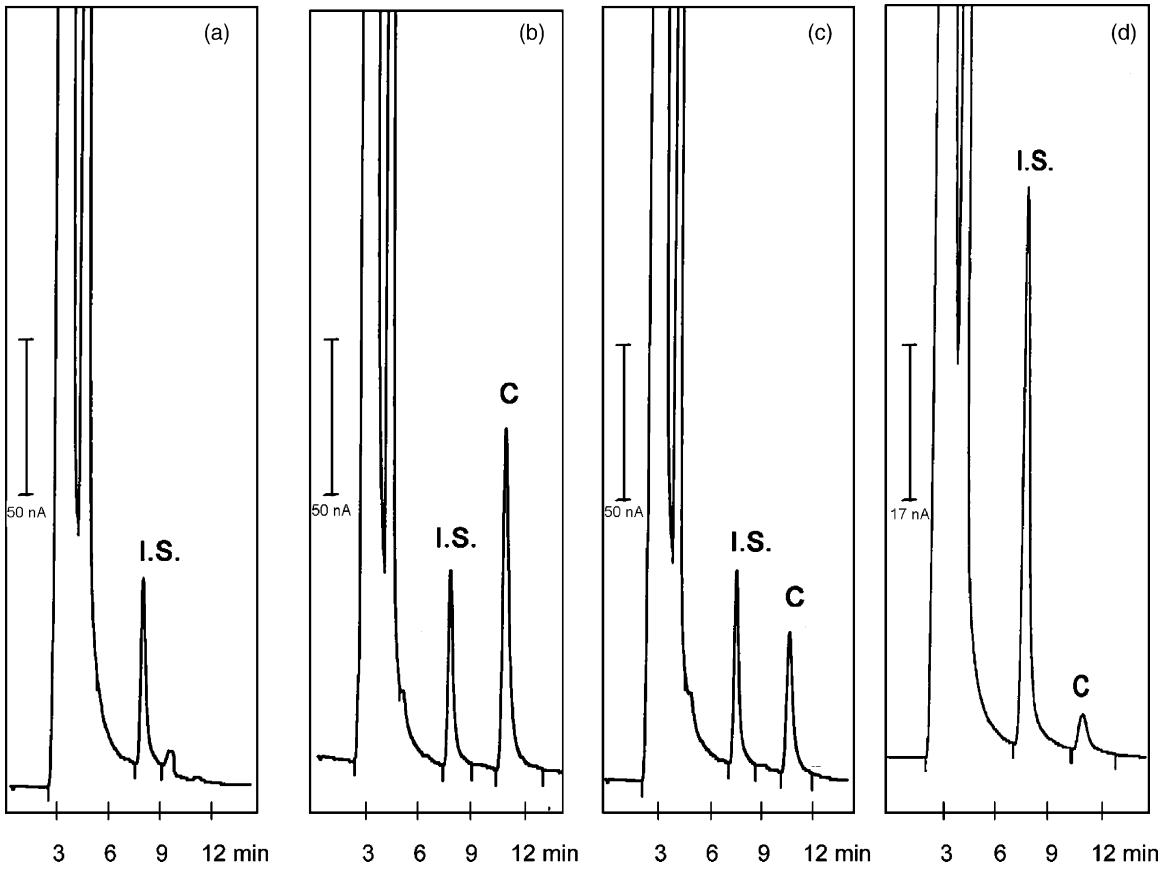

Fig. 1. Plasma extracts (a) plasma blank: IS 40 ng; (b) plasma standard: clioquinol 133 ng/ml, IS 40 ng; (c) plasma sample: clioquinol 60 ng/ml, IS 40 ng and (d) LOQ, clioquinol 5 ng/ml, IS 40 ng.

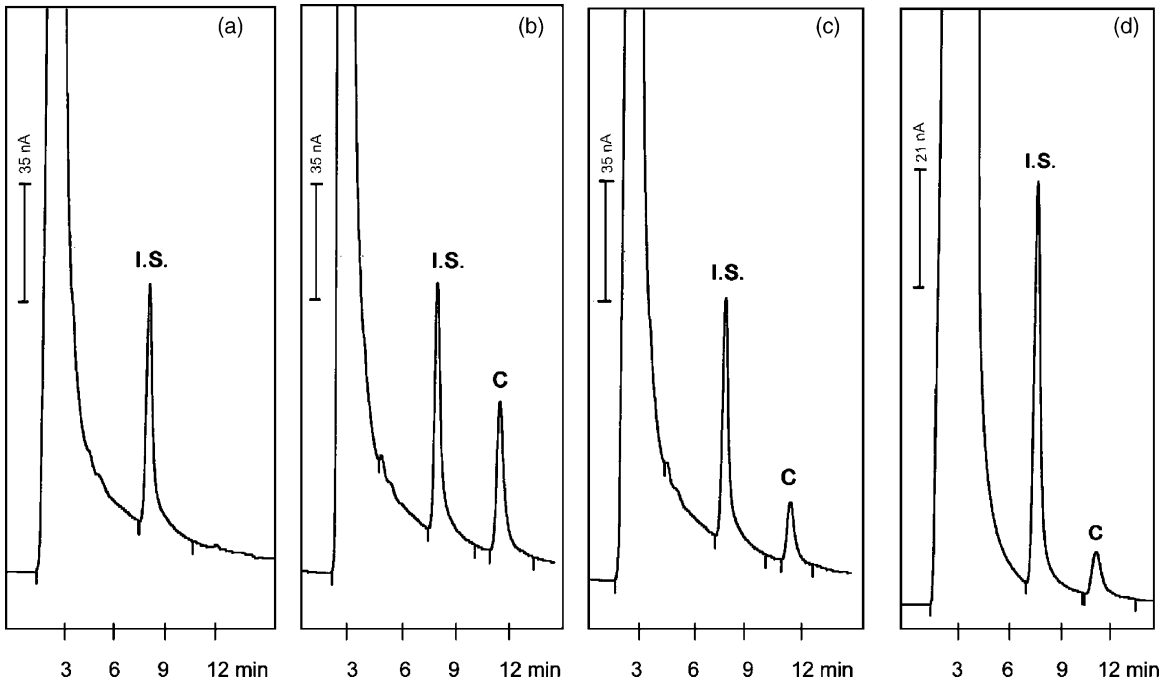

Fig. 2. Brain extracts (a) brain tissue blank: IS 120 ng; (b) brain tissue standard: clioquinol 60 ng/ml, IS 120 ng; (c) brain tissue sample: clioquinol 24 ng/ml, IS 120 ng and (d) LOQ, clioquinol 10 ng/ml, IS 120 ng.

2.7. Recovery

Absolute clioquinol recovery from the plasma and tissue samples was determined by comparing the areas of the peaks of the unextracted standards with those of the standards extracted as described above.

3. Results

3.1. Determination of clioquinol in plasma and tissue samples

Under our conditions, the clioquinol and IS retention times were, respectively, 11.6 and 8.1 min. There were no interfering peaks near the retention times of the compounds in the chromatograms of the blank or the baseline (time 0) plasma and tissue samples. Selectivity was calculated as $[\Delta t/(1/2\ Wa + Wb)]$, where W are the bandwidths of the peaks, and found to be 11.1 (IS versus C).

As there is no acknowledged treatment for TSE, the possible interference of co-administered anti-cholinesterase compounds was tested as these are standard treatments in patients with Alzheimer's disease who may receive clioquinol in clinical trials. Under our conditions, none of these compounds (tacrine, rivastigmine, donepezil and galanthamine) were detectable by the present method, and thus did not interfere with the assay of clioquinol. Figs. 1 and 2 show representative chromatograms.

3.2. Linearity

Six standard samples of clioquinol 5–2000 ng/ml in plasma and 10–1000 ng/g in tissues were analysed in triplicate in order

Table 1
Precision, accuracy and recovery of clioquinol determinations in plasma and tissues

Nominal concentrations (ng/ml)	Intra-assay precision (CV%) ($n = 10$)	Inter-assay precision (CV%) ($n = 6$)	Intra-assay accuracy (%) ($n = 10$)	Inter-assay accuracy (%) ($n = 6$)	Recovery (%) (M ± S.D.)
Plasma					
10	6.4	7.0	95.7	94.4	75.1 ± 3.7
100	3.0	3.5	96.6	99.5	76.8 ± 2.7
1000	3.8	4.0	99.8	100.3	75.6 ± 3.0
Brain					
20	7.1	7.7	94.1	95.0	72.3 ± 3.9
100	4.0	4.5	98.3	98.2	75.1 ± 3.6
500	3.8	4.0	95.7	97.9	74.1 ± 3.6
Liver					
20	7.0	7.5	96.1	95.0	73.3 ± 3.2
100	3.8	4.8	98.5	98.5	74.1 ± 4.1
500	3.0	4.1	99.9	99.2	76.2 ± 3.5

Table 2
Unconjugated and total clioquinol concentrations in plasma and brain 60 min after p.o. administration ($n = 6$)

	Unconjugated clioquinol concentrations (M ± S.D.)	Total clioquinol concentrations (M ± S.D.)
Plasma (ng/ml)	983 ± 145	1261 ± 197
Brain (ng/g)	81 ± 26	–
Liver (ng/g)	395 ± 92	–

to determine the linearity of the assay. The ratio of the area of the peak of clioquinol versus the IS was linearly related ($y = 0.68$ (±0.11, S.D.) +1.08 (±0.018) x, $r = 0.994$, range: 0.991–0.996, for plasma; $y = 0.02$ (±0.03) +1.42 (±0.02) x, $r = 0.993$, range: 0.992–0.997, for tissues) to drug concentrations within the studied range.

3.3. Accuracy and precision

The mean accuracy of the intra-day clioquinol assay was 97.4% in plasma, and 96.0% in brain, with a mean precision (CV) of respectively, 4.4 and 5.0%; the mean accuracy of the inter-day clioquinol assay was 98.1% in plasma and 97.0% in brain, with a mean precision (CV) of respectively, 4.8 and 5.4% (Table 1). These results validated the calibration curves used for each set of samples. The results obtained using the quality control samples kept at −25 °C for 2 weeks ensured sample stability under our conditions (see the inter-day accuracy above).

3.4. Sensitivity and recovery

The lower limit of clioquinol detection (a signal that was three times that of the blank) was 2 ng/ml in plasma and 5 ng/ml in tissues, and the lower limit of quantitation (CV ≤ 8%) was 5 ng/ml in plasma and 10 ng/ml in tissues.

The mean absolute recovery of clioquinol determined by comparing the areas of the peaks of the unextracted standards with those of the standards extracted as described above was higher than 75% in plasma and 72% in tissue (Table 1).

3.5. Clioquinol concentrations in biological samples

Table 2 shows the plasma, brain and spleen concentrations of clioquinol 60 min after the oral administration of a 50 mg/kg dose.

4. Conclusions

We here describe an analytical method for extracting and quantitating clioquinol in hamster plasma and tissues, which makes it possible to study the pharmacokinetics of clioquinol in plasma after intraperitoneal and oral administration [22].

In comparison with previous methods [13–17], the selectivity of electrochemical detection avoids the need for complex extraction procedures and greatly improves sensitivity over UV detection (300 ng/ml) [13,17] or 200 ng/ml [18] and GC/electron capture (50 ng/ml) [19]. The sensitivity of the GC–MS method of Matsuki et al. [20] has a sensitivity of 0.10 ng as the minimum amount injected into the chromatograph which is similar to that of the present method (0.15 ng) but requires a rather complex sample preparation procedure and expensive instruments that are not available in all laboratories.

The sensitivity, selectivity and simplicity of the method also make it a valuable means of monitoring clioquinol levels in treated patients, as in recent studies of patients with Alzheimer's disease [1], insofar as our preliminary data concerning human plasma show that the plasma blanks and sensitivity are similar to those found when using hamster plasma, and there was no interference with the drugs used as standard therapies in such patients.

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