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# High-performance liquid chromatography measurement of hyperforin and its reduced derivatives in rodent plasma

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**Abstract**

A reverse-phase high-performance liquid chromatography method was developed for the determination of hyperforin and its reduced derivatives octahydrohyperforin and tetrahydrohyperforin in rodent plasma. The procedure includes solid-phase extraction from plasma using the Baker 3cc C8 cartridge, resolution on the Symmetry Shield RP8 column (150 mm × 4.6 mm, i.d. 3.5 μm) and UV absorbance detection at 300 nm. The assay was linear over a wide range, with an overall coefficient of variation less than 10% for all compounds. The precision and accuracy were within acceptable limits and the limit of quantitation was sufficient for studies preliminarily assessing the disposition of tetrahydrohyperforin and octahydrohyperforin in the mouse and rat.


*Keywords:* Hyperforin; Octahydrohyperforin; Tetrahydrohyperforin; Pharmacokinetic; Rodents

## 1. Introduction

Recent studies [1–5] have aimed at identifying the constituents that account for the pharmacological effect of *Hypericum perforatum* extracts, because of their widespread, mostly unregulated use for the treatment of mild to moderate depression. Although several components have antidepressant-like effects in animals, evidence is growing that hyperforin plays a decisive role. This acylphloroglucinol derivative interacts with several neurotransmitter mechanisms believed to be causally involved in depression [6], although at concentrations generally higher than those achieved in rodent brain after doses active in behavioural tests predictive of antidepressant activity [4]. Hyperforin shares most of the neuropharmacological properties of *H. perforatum* extracts, and the pharmacological potencies of different extracts correlate with their hyperforin content [1,2,7,8]. Hyperforin, however, is poorly stable when extracted from the herb and exposed to light and air [9,10], and its liability to oxidation complicates the preparation of pharmaceutical and nutritional formulations of *H. perforatum* extracts whose pharmacological activities may be rapidly lost during storage [11]. This has led to the synthesis and pharmacological evaluation of more stable analogues, including various esters, salts and hydroxy-functionalized derivatives of hyperforin [12–15].

Reduced derivatives of hyperforin, obtained by catalytic reduction of the double bonds of the isoprene chain and/or reduction of the keto groups at the 1- and 10-positions [16], have also been studied. Some of these compounds not only have high stability, but their antidepressant-like activity in rats is surprisingly higher than hyperforin [17]. They are therefore undergoing pre-clinical studies in rodents, including an evaluation of their inductive potential compared to hyperforin, which potently activates the human pregnane X receptor, thus causing several clinically relevant interactions involving *H. perforatum* extracts and substrates of CYP3A [18]. Measurements of their plasma concentrations in animal models may help in assessing the pharmacological importance of potential adverse findings and in extrapolating pharmacolog-

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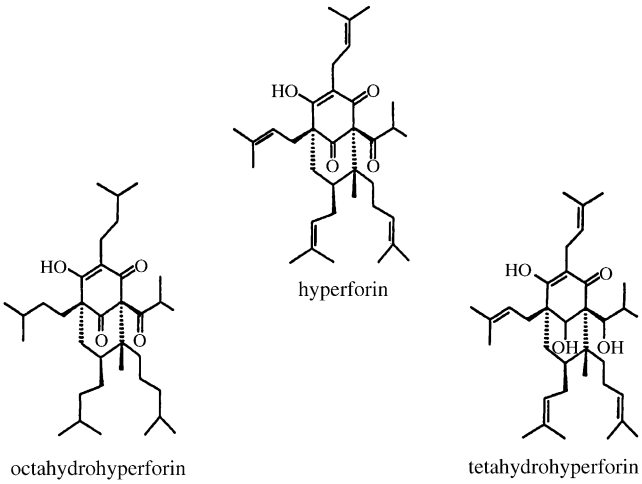


Fig. 1. Chemical structures of hyperforin and its reduced derivatives octahydrohyperforin and tetrahydrohyperforin.

ical data across species. This obviously calls for a fast and sensitive analytical method to quantitate hyperforin and its chemically related compounds.

Hyperforin has been measured in plasma and tissues by selective and sensitive chromatographic procedures such as high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [4,19–22] or coupled with tandem mass spectrometry (LC/MS) [23,24]. The LC/MS procedures are particularly sensitive but their application is limited on account of the instrument availability. The HPLC-UV methods can be routinely used in all laboratories, being based on simple liquid–liquid extraction and reverse-phase chromatography, although some have disadvantages such as the use of polar solvents [20] that could potentially affect hyperforin's stability [21] or detection limits too high for application in pharmacokinetic studies [19].

As a first step to support pharmacological studies of hyperforin analogues we therefore modified some reported methods for measuring hyperforin [4,19,20], in order to facilitate extraction, improve sensitivity and allow quantitation of the hyperforin derivatives not previously studied. The procedure still relies on solid-phase extraction but uses an octyl column to extract these non-polar compounds from plasma which are then resolved from the internal standard (I.S.) and endogenous components on a RP8 reverse-phase column. We have used this method to obtain preliminary data on the single and repeated oral pharmacokinetics of octahydrohyperforin and tetrahydrohyperforin in the mouse and rat. The structures of hyperforin and its reduced derivatives are shown in Fig. 1.

## 2. Experimental

### 2.1. Chemicals

Hyperforin (as the dicyclohexylammonium salt; DCHA), tetrahydrohyperforin, octahydrohyperforin and its lithium salt (octahydrohyperforin Li) were kindly supplied by Indena S.p.A (Milan, Italy). Stock solutions were prepared in amber-coloured volumetric flasks by dissolving the compounds in methanol at a concentration of 1 mg/ml. Working standards were prepared from the stock solutions by dilution with methanol and kept at −4 °C. Drug-free plasma for the preparation of the calibration standards and quality control samples (QC) were obtained from male CD1 mice and male Sprague-Dawley rats (Charles River, Italy). Plasma was stored at −20 °C until analysis.

Other chemicals and solvents were of analytical-reagent grade and were used without further purification. Water was deionised and distilled before use.

### 2.2. Chromatographic apparatus and conditions

HPLC analysis was done on a Waters system equipped with an Autosampler Waters 712 processor, a Model 600 solvent delivery system and a Model 996 UV detector controlled by Millennium$^{32}$ software (Waters Milford, MA, USA).

Separation was on a Symmetry Shield RP8 column (150 mm × 4.6 mm, i.d. 3.5 μm) protected by a Symmetry Shield RP8—5 μm precolumn (Waters Milford, MA, USA), at room temperature. The mobile phase was methanol: acetonitrile:*n*-butanol:0.01 M phosphate buffer pH 7.4 (55:18:1:26, v/v). The eluent was filtered through a 0.45-μm filter degassed before use and delivered isocratically at a flow-rate of 0.8 ml/min.

### 2.3. Extraction

Baker 3cc (200 mg) C8 cartridges (Mallinckrodt Baker, Phillipburg, USA) were used to clean up plasma samples. The cartridges were pre-wetted with 2 ml of $CH_3CN$ and 2 ml of distilled water. Then, after adding the I.S., plasma samples (0.3–1 ml), diluted to 0.6–2 ml with 0.01 M phosphate buffer pH 7.4–acetonitrile (30:70, v/v) and centrifuged at 5000 rpm for 10 min at 4 °C, were added and the cartridges were washed with 2 ml distilled water and 0.2 ml of acetonitrile, interrupting the vacuum before reaching dryness of the column after each passage. The compound was removed by eluting the cartridges with 2 ml of acetonitrile and evaporated to dryness under nitrogen flow. The residue was dissolved in the mobile phase (200 μl), centrifuged at 5000 rpm for 10 min, and 170 μl were analysed by HPLC with UV detection (300 nm).

### 2.4. Assay calibration and performance

Standard calibration graphs were constructed by linear least-squares regression analysis of the plot of the peak-height ratio between the analyte and the I.S. response, against the concentrations in the standard samples [25]. Computer-generated parameters were used to convert the relative response of the unknown samples to concentrations.

Three standard curves with seven concentrations of hyperforin and its derivatives were analysed concurrently with

each set of QC, and unknown samples. The lowest calibration standard corresponded to the limit of quantification (LOQ), i.e. the lowest concentration that could be measured with acceptable accuracy and precision (≤20%), as determined in separate studies. The upper limit of quantitation was arbitrarily defined as 1.66 μg ml$^{-1}$ (hyperforin and tetrahydrohyperforin) and 3.33 μg ml$^{-1}$ (octahydrohyperforin), using 0.3 ml of plasma.

The quality of the analytical results was checked by three replicate analyses of QC containing small (0.033 μg ml$^{-1}$ using 0.3 ml of plasma), medium (0.167 μg ml$^{-1}$) and large (0.833 μg ml$^{-1}$) amounts of hyperforin and tetrahydrohyperforin, and QC containing small (0.083 μg ml$^{-1}$ using 0.3 ml of plasma), medium (0.333 μg ml$^{-1}$) and large (1.665 μg ml$^{-1}$) amounts of octahydrohyperforin, stored at −20 °C. On three different days these QC were assayed with standard samples and the calculated concentrations were compared (inter-assay variance). Intra-assay variance was checked by three replicate analysis of QC samples on the same day.

### 2.5. Determination of stability

To verify the stability of hyperforin derivatives in rat plasma, aliquots of the standard solutions of octahydrohyperforin (0.083–1.665 μg ml$^{-1}$) and tetrahydrohyperforin (0.033 and 0.833 μg ml$^{-1}$) were spiked into extraction tubes containing fresh plasma and tissue homogenate. The tubes were stored at room temperature or at −20 °C before analysis. The tubes stored at room temperature were analyzed after 1 h, and those at −20 °C were analyzed 1 month later to check the long-term stability of octahydrohyperforin. The analytical response of the stored samples was compared with samples prepared on the day of analysis.

The stability of the three compounds in the mobile phase in the autosampler at room temperature was assessed by repeated injection of spiked plasma and brain samples for 24 h.

### 2.6. In vivo studies

Adult male CD-COBS mice (18–20 g) and rats (175–200 g) (Charles River, Calco, Italy) were given octahydrohyperforin Li or tetrahydrohyperforin orally, suspended in 4% Tween 80 in water. Animals were then killed by decapitation at various times thereafter; blood was collected in heparinized tubes and centrifuged to separate plasma which was stored at −20 °C until analysis.

Plasma concentration–time data were analyzed by classical model-independent methods. The area under the plasma concentration–time curve from zero to the last measurable concentration ($AUC_t$) was determined by the trapezoidal rule. The observed maximum plasma concentration ($C_{max}$) and the time of its occurrence ($t_{max}$) were read directly from the plasma and brain concentration–time data.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116. G.U., suppl. 40. 18 Febbraio 1992, Circolare No. 8. G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609. OJ L 358.1, Dec. 12. 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

## 3. Results and discussion

### 3.1. Development of the method

Currently the most convenient method for extraction of hyperforin from plasma and homogenised tissues is solid phase extraction which has the advantage of not needing any nonpolar solvents that might affect hyperforin stability [21], besides being simple and effective in removing interference from endogenous components. Standard octyl extraction cartridges, which are typically used in a reverse phase mode for separation of non-ionic, non-polar to moderately polar compounds were therefore tested for extracting the reduced derivatives tetrahydrohyperforin and octahydrohyperforin from mouse and rat plasma. The best yields for all compounds were with the Baker 3cc (200 mg) C8 cartridge, eluting with $CH_3CN$ which was therefore used as described for hyperforin [4]. This procedure extracted only a few impurities and no interfering compounds, regardless of the species or volume of plasma considered. Mean overall recoveries, determined by comparing the peak height of the analyte from spiked mouse and rat plasma (0.3–1 ml) with those from direct injection of the compounds dissolved in the mobile phase, averaged 87 ± 7% for hyperforin and 84 ± 9% and 83 ± 8% for the tetrahydro- and octahydro-derivatives, respectively (Table 1).

Fig. 2 gives examples of chromatograms of extracts from drug-free plasma (A), spiked plasma (B) and plasma of a mouse given orally 18.4 mg kg$^{-1}$ octahydrohyperforin (C) or tetrahydrohyperforin (D) and killed 1 h after dosing. Separation was on the Symmetry Shield RP8 column with the mobile phase consisting of methanol:acetonitrile:*n*-butanol:0.01 M phosphate buffer pH 7.4 (55:18:1:26, v/v). Hyperforin (retention time 8 min) was an acceptable I.S. in the analysis of both reduced derivatives, although tetrahydrohyperforin (13.5 min) can be used in the analysis of octahydrohyperforin (17.5 min). Either tetrahydrohyperforin or octahydrohyperforin can obviously be used as I.S. for hyperforin quantification, although the former was used in this study because its retention time is closer to hyperforin, shortening chromatographic analysis time. Tetrahydrohyperforin and octahydrohyperforin were separated from hyperforin and endogenous substances also on the X-Terra RP8 (150 mm × 4.6 mm, 5.0 μm) and Zorbax C8 (150 mm × 4.6 mm, 5.0 μm) columns, although on this latter the reduced derivatives eluted together, with the present mobile phase (data not shown).

The relationships between the peak-height ratios of each derivative to the I.S. and the amount of the compound added

Table 1
Mean recoveries of hyperforin and its reduced derivatives from spiked plasma

| Tissue (ml) | Compound | Concentrations ($\mu g\,ml^{-1}$) | Recovery (% ± S.D.) |
|---|---|---|---|
| Mouse plasma (0.3) | Hyperforin | 0.03–0.83 | 85 ± 6 |
| | Tetrahydrohyperforin | 0.03–0.83 | 80 ± 8 |
| | Octahydrohyperforin | 0.08–1.66 | 82 ± 11 |
| Rat plasma (1) | Hyperforin | 0.01–0.25 | 90 ± 9 |
| | Tetrahydrohyperforin | 0.01–0.25 | 88 ± 10 |
| | Octahydrohyperforin | 0.03–0.50 | 85 ± 5 |

to plasma were always linear, with a correlation coefficient invariably exceeding 0.9995. The slopes of three curves prepared on three different days had a coefficient of variation (C.V.) of 4.9% and 2.2% and average regression equation $y = 0.0123x + 0.0215$ and $y = 0.0060x + 0.0220$ respectively for tetrahydrohyperforin and octahydrohyperforin. The lowest calibration standard corresponded to the LOQ and was $0.017\,\mu g\,ml^{-1}$ for tetrahydrohyperforin (and hyperforin) and $0.033\,\mu g\,ml^{-1}$ for octahydrohyperforin, using 0.3 ml of plasma. At these concentrations the C.V. for the precision and reproducibility of the assay were below 10% for all derivatives.

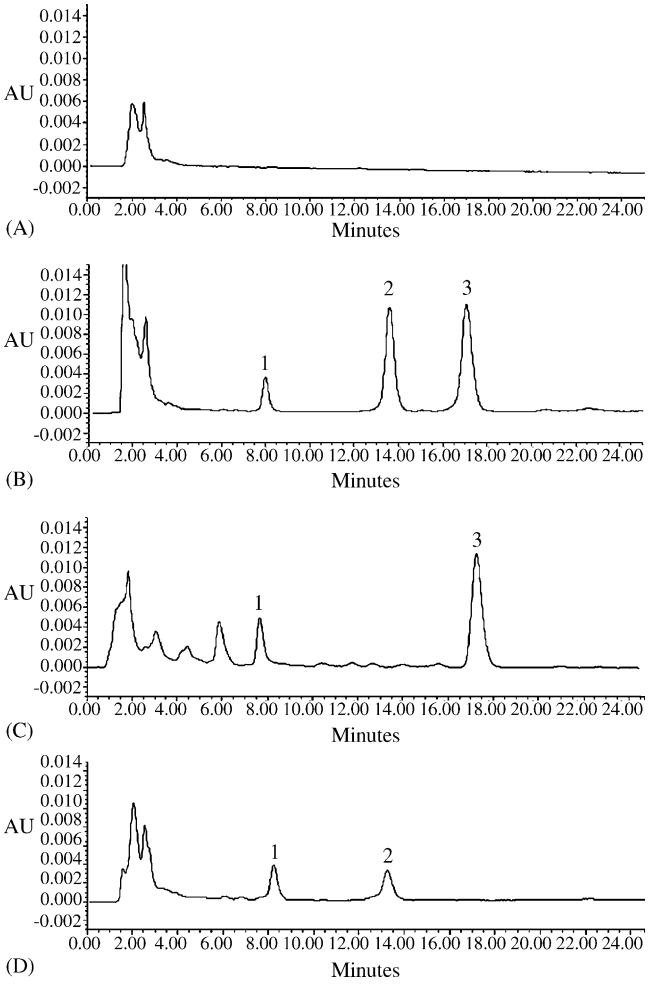


Fig. 2. Chromatograms of extracts from drug-free mouse plasma (A) and (B) plasma spiked with $0.167\,\mu g\,ml^{-1}$ hyperforin (1), $0.833\,\mu g\,ml^{-1}$ tetrahydrohyperforin (2) and $1.665\,\mu g\,ml^{-1}$ octahydrohyperforin (3). Also shown plasma samples from mice given orally $18.4\,mg\,kg^{-1}$ of octahydrohyperforin (C) or tetrahydrohyperforin (D). Column: Symmetry Shield RP8 column (150 mm × 4.6 mm, i.d. 3.5 μm). Mobile phase, methanol:acetonitrile:*n*-butanol:0.01 M phosphate buffer, pH 7.4 (55:18:1:26, v/v).

The reproducibility of the method was evaluated analyzing three replicates of QC samples containing the compound at the nominal concentrations of 0.033, 0.167 and $0.833\,\mu g\,ml^{-1}$ for hyperforin and tetrahydrohyperforin and 0.083, 0.333 and $1.665\,\mu g\,ml^{-1}$ for octahydrohyperforin on three different days. The intra- and inter-day precision (expressed as C.V.) and accuracy (R.E.) are reported in Table 2. The method was found to be precise within acceptable limits, with C.V. $\leq 10.6\%$, $\leq 7.1\%$ and $\leq 5.0\%$ for hyperforin, tetrahydrohyperforin and octahydrohyperforin respectively, and R.E. ranging from −1.7 to 1.3% for hyperforin, from −6.0 to 4.1% for tetrahydrohyperforin and from −3.6 to

Table 2
Summary of intra- and inter-assay precision and accuracy data in QC samples

| | Added ($\mu g\,ml^{-1}$) | Mean observed | CV% | R.E.[a] |
|---|---|---|---|---|
| Hyperforin | | | | |
| Within-day ($n = 3$) | 0.033 | 0.033 | 3.585 | 0 |
| | 0.167 | 0.164 | 9.982 | −1.7 |
| | 0.833 | 0.820 | 1.191 | −1.5 |
| Day to day ($n = 3$) | 0.033 | 0.033 | 4.911 | 0 |
| | 0.167 | 0.167 | 10.667 | 0 |
| | 0.833 | 0.844 | 5.468 | 1.3 |
| Tetrahydrohyperforin | | | | |
| Within-day ($n = 3$) | 0.033 | 0.031 | 1.576 | −6.0 |
| | 0.167 | 0.170 | 3.138 | 1.7 |
| | 0.833 | 0.849 | 6.092 | 1.9 |
| Day to day ($n = 3$) | 0.033 | 0.034 | 7.167 | 3.0 |
| | 0.167 | 0.174 | 6.060 | 4.1 |
| | 0.833 | 0.848 | 4.253 | 1.8 |
| Octahydrohyperforin | | | | |
| Within-day ($n = 3$) | 0.083 | 0.084 | 1.956 | 1.2 |
| | 0.333 | 0.321 | 2.720 | −3.6 |
| | 1.665 | 1.640 | 1.819 | −1.5 |
| Day to day ($n = 3$) | 0.083 | 0.083 | 4.862 | |
| | 0.333 | 0.340 | 5.050 | 3.0 |
| | 1.665 | 1.684 | 2.758 | 1.1 |

[a] R.E. = (calculated − nominal/nominal) × 100.

3.0 for octahydrohyperforin, in the range of concentrations tested.

These compounds were stable for at least 1 h at room temperature in plasma samples; the amount was 102 ± 4% of the original concentration for tetrahydrohyperforin (0.033 and 0.833 μg ml$^{-1}$), and 102 ± 2% for octahydrohyperforin (0.083 and 1.665 μg ml$^{-1}$). Hyperforin derivatives appear stable in frozen mouse plasma; 95 ± 6% of the original concentration of tetrahydrohyperforin (0.033 and 0.833 μg ml$^{-1}$) was found in the QC samples after 1 month at −20 °C. Octahydrohyperforin (0.083 and 1.665 μg ml$^{-1}$) was stable too in these conditions; 94 ± 6% of the nominal concentration of the compound added to QC samples was found.

### 3.2. Drug measurements

The HPLC procedure was used preliminarily to assess exposure to reduced derivatives of hyperforin after single and repeated oral doses in rodents. Fig. 3 shows the plasma concentration–time curves of octahydrohyperforin and tetrahydrohyperforin in rats given 30 mg kg$^{-1}$ of the compounds (as Li salt, in the case of octahydrohyperforin). Octahydrohyperforin appeared rapidly, achieving mean plasma $C_{max}$ (553 ng ml$^{-1}$) at 30 min, i.e. the first sampling time. The elimination of the compound from plasma was relatively slow thereafter, with quantifiable levels up to 24 h, although they were only just above the limit of detection. Tetrahydrohyperforin plasma $C_{max}$ occurred within 60–120 min and amounted to about 211 ng ml$^{-1}$; these concentrations rapidly declined close to the LOQ at 8 h, with the limitation dictated by the small number of points and animals

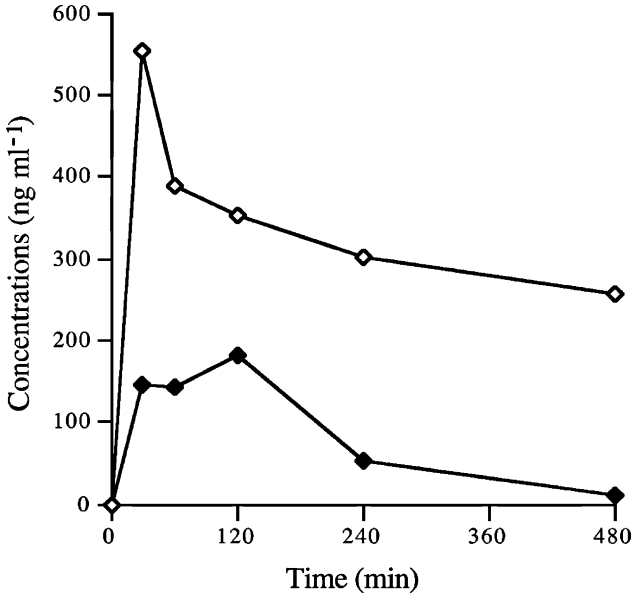


Fig. 3. Mean plasma concentration–time curves of octahydrohyperforin (open symbols) and tetrahydrohyperforin (closed symbols) after an oral dose of 30 mg kg$^{-1}$ to two rats/group.

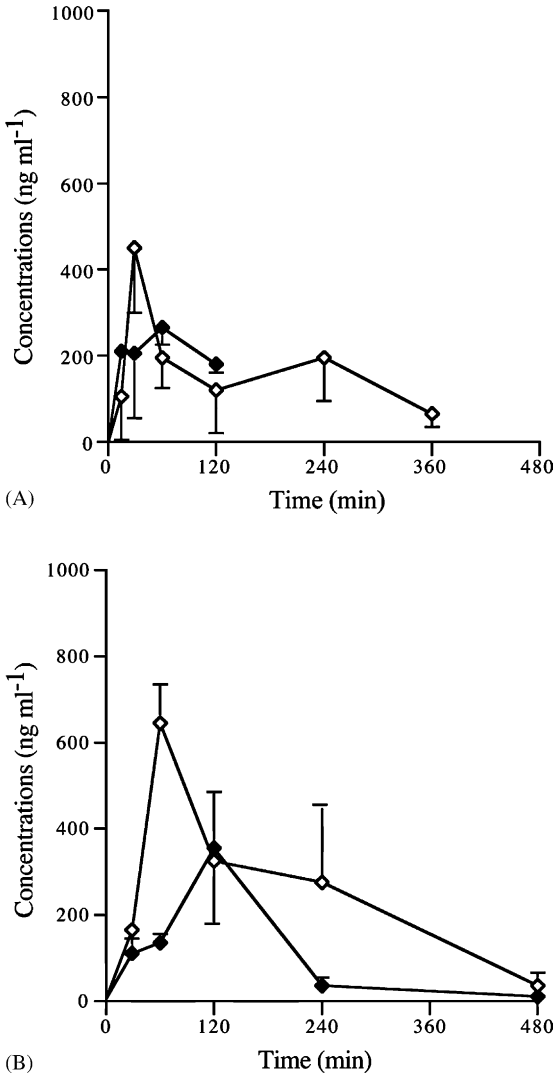


Fig. 4. Mean plasma concentration–time curves of hyperforin (A) and octahydrohyperforin (B) after the first (open symbols) and the last (closed symbols) dose in mice (a twice daily oral regimen of 18.1 and 18.4 mg kg$^{-1}$, respectively). Each point is the mean ± S.D. of 3–6 mice.

examined. Mean plasma $AUC_{0-8\,h}$ averaged 635 ng ml$^{-1}$ h for tetrahydrohyperforin and 2521 ng ml$^{-1}$ h for octahydrohyperforin.

Fig. 4B compares the plasma concentration–time profile of octahydrohyperforin after the first and the last dose of a twice-daily regimen of 18.4 mg kg$^{-1}$ octahydrohyperforin Li for 3 days in mice. This dose was equimolar to hyperforin 18.1 mg kg$^{-1}$ (Fig. 4A) which approximated the hyperforin content in an *H. perforatum* extract (300 mg kg$^{-1}$) that induced the activity and expression of CYP3A proteins in the mouse. Like hyperforin [26], octahydrohyperforin maximum plasma concentrations were variable and tended to be lower after the last dose of this schedule (131 ng ml$^{-1}$ versus 645 ng ml$^{-1}$). Partial plasma AUC approximately halved after the last doses (791 ng ml$^{-1}$ h versus 1931 ng ml$^{-1}$ h), suggesting that the metabolism of this compound involves oxidative reactions which may be autoinduced by repeated

dosing. However, the activity of the CYP3A-dependent erythromycin *N*-demethylase was not significantly increased (L. Cantoni, personal communication) suggesting that octahydrohyperforin behaves differently from hyperforin as regard the pattern and potential for induction of CYP enzymes. Because of species differences in the response of inducible enzymes, definitive risk assessment of the potential induction of CYP enzymes by this and other hyperforin derivatives should come from in vitro and in vivo human studies.

Preliminary brain-to-blood distribution studies were done in the mouse and rat because of potential species differences in brain uptake of antidepressant drugs and centrally acting drugs in general [27]. The procedure was as for hyperforin [4]; after homogenization (5 ml $g^{-1}$) in 0.01 M phosphate buffer, pH 7.4–$CH_3CN$ (30–70%, v/v), and centrifugation, the supernatant (1.5 ml for mouse brain and 2 ml for rat brain) was processed as for plasma. The assay was satisfactory in terms of recovery and specificity, with a LOQ of 0.017 μg $g^{-1}$ for tetrahydrohyperforin and 0.033 μg/g for octahydrohyperforin in mice, 0.025 μg $g^{-1}$ for tetrahydro- and octahydrohyperforin in rats, and an overall C.V. for intra-assay precision of less than 10% for both derivatives (inter-day precision was not evaluated). However, the two compounds could not be quantified in brain tissue from rats given single oral doses of tetrahydrohyperforin and octahydrohyperforin (30 mg $kg^{-1}$), because the whole-brain concentrations were always below the LOQ of the procedure within 24 h of oral dosing. The same was true after repeated oral doses (18.4 mg $kg^{-1}$, twice daily) of octahydrohyperforin in mice suggesting that the exchange between blood and brain is relatively restricted for this class of potential antidepressants.

## 4. Conclusions

The search for more stable and safer analogs of hyperforin has led to the pharmacological evaluation of its reduced derivatives including tetrahydrohyperforin and octahydrohyperforin, and some of their salts and esters which appears particularly effective in animal tests predictive of antidepressant activity. Pharmacokinetic studies may help in rationalising these studies, mostly done in vitro and in vivo in rodents. An appropriate analytical method for quantification of all these derivatives, like the one proposed here, is a pre-requisite. This procedure requires only a small volume of plasma, is precise and reproducible, with a LOQ sufficient for pharmacokinetic studies in rodents, though even lower limits could be achieved using higher volumes of plasma.

The sensitivity of the method did not allow measurements of hyperforin and its reduced derivatives in the central nervous system but this may not be a limiting factor in the evaluation of their disposition. Hyperforin brain concentrations of only 15–30 ng $g^{-1}$ (0.03–0.06 μM, assuming 1 g brain tissue equivalent to 1 ml water) were found in mouse brain after pharmacologically effective doses of the compound either as the sodium salt or as about 5% *H. perforatum* extract [24], suggesting poor passage of the blood-brain barrier. Accordingly, hyperforin brain concentrations (0.06 μM) amounted to only 4% of the plasma concentrations (1.4 μM) in rats given three 12.5 mg $kg^{-1}$ injections of hyperforin DCHA [4], and were possibly entirely related to hyperforin's effective contribution from blood, allowing for at least 10 μl $g^{-1}$ of residual blood in the brain [28]. This suggested that the antidepressant-like activities of hyperforin are not due to a direct interaction with neurotransmitter mechanisms believed to be involved in the central effects of common synthetic antidepressants [4]. This may also be true for octahydrohyperforin and tetrahydrohyperforin which, like hyperforin, interact in vitro with several central neurotransmitter receptors and transporters at concentrations far exceeding (>0.5–1 μM; M. Gobbi, personal communication) those reached in vivo after relatively high oral doses in rodents (<30 ng $g^{-1}$ or 0.06 μM, using about 300 mg of brain tissue).

## References

[1] S.K. Bhattacharya, A. Chakrabarti, S.S. Chatterjee, Pharmacopsychiatry 31 (1998) 22.
[2] S.S. chatterjee, S.K. Bhattacharya, M. Wonnemann, A. Singer, W.E. Muller, Life Sci. 63 (1998) 499.
[3] A.G. Jensen, S.H. Hansen, E.Ø. Nielsen, Life Sci. 68 (2001) 1593.
[4] L. Cervo, M. Rozio, C.B. Ekalle-Soppo, G. Guiso, P. Morazzoni, S. Caccia, Psychopharmacology (Berlin) 164 (2002) 423.
[5] V. Butterweck, V. Christoffel, A. Nahrstedt, F. Petereit, B. Spengler, H. Winterhoff, Life Sci. 9402 (2003) 1.
[6] J.M. Greeson, B. Sanford, D.A. Monti, Psychopharmacology (Berlin) 153 (2001) 402.
[7] S.S. Chatterjee, M. Noldner, E. Koch, C. Erdelmeier, Pharmacopsychiatry 31 (Suppl. 1) (1998) 7.
[8] G. Laakmann, C. Schule, T. Baghai, M. Kieser, Pharmacopsychiatry 31 (1998) 54.
[9] C.A. Erdelmeier, Pharmacopsychiatry 31 (Suppl. 1) (1998) 2.
[10] A.R. Bilia, M.C. Bergonzi, F. Morgenni, G. Mazzi, F.F. Vincieri, Int. J. Pharm. 213 (2001) 199.
[11] L.G. Guilhermano, L. Ortiz, M. Ferigolo, H.M.T. Barros, Prog. Neuropsychopharmacol. Biol. Psychiatry 28 (2004) 49.
[12] P. Zanoli, M. Rivasi, C. Baraldi, M. Baraldi, Behav. Pharmacol. 13 (2002) 645.
[13] International Publication Number: WO 99/41220, International Application Number: PCT/EP99/00737, International Publication Date: 19 August 1999.
[14] International Publication Number: WO 99/64388, International Application Number: PCT/EP99/03880, International Publication Date: 16 December 1999.
[15] E. Bombardelli, P. Morazzoni, A. Riva, Eur. Neuropsycopharmacol. 3 (Suppl. 12) (2002) S240.
[16] N.S. Bystrov, V.N. Dobrynin, M.N. Kolosov, S.A. Popravko, B.K. Chernov, Bioorg. Khim. 4 (6) (1978) 791.
[17] International Publication Number: WO 03/091194, International Application Number: PCT/EP03/04100, International Publication Date: 6 November 2003.
[18] C. Ioannides, Xenobiotica 32 (2002) 451.
[19] J.D. Chi, M. Franklin, J. Chromatogr. B 735 (1999) 285.
[20] S. Bauer, E. Stormer, H.-J. Graubaum, I. Roots, J. Chromatogr. B 765 (2001) 29.
[21] Y. Cui, B. Gurley, C.Y.W. Ang, J. Leakey, J. Chromatogr. B 780 (2002) 129.

[22] R. Pirker, C.W. Huck, G.K. Bonn, J. Chromatogr. B 777 (2002) 147.
[23] A. Biber, H. Fischer, A. Romer, S.S. Chatterjee, Pharmacopsychiatry 31 (Supplement) (1998) 36.
[24] J.H. Keller, M. Karas, W.E. Muller, D.A. Volmer, G.P. Eckert, M.A. Tawab, H.H. Blume, T. Dingermann, M. Schubert-Zsilavecz, Anal. Chem. 75 (2003) 6084.
[25] R. Causon, J. Chromatogr. B 689 (1997) 175.
[26] L. Cantoni, M. Rozio, A. Mangolini, L. Hauri, S. Caccia, Toxicol. Sci. 75 (2003) 25.
[27] S. Caccia, Clin. Pharmacokin. 34 (1998) 281.
[28] M.A. Gonzales, T.N. Tozer, D.T.T. Chang, J. Pharm. Sci. 64 (1975) 99.