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Short communication

Determination of hypericin in plasma by high-performance liquid chromatography

J.D. Chi*, M. Franklin

University of Oxford, Department of Psychiatry, Psychopharmacology Research Unit, Warneford Hospital, Oxford, OX3 7JX, UK

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Abstract

Hypericin, a polycyclic dianthroquinone, is one of the characteristic ingredients of *Hypericum perforatum* extracts (St. John′s wort, HP), which has antidepressant effects. Hypericin and the internal standard (I.S.), dansylamide, were extracted from plasma utilizing solid-phase extraction (SPE). Chromatography was performed using isocratic reversed-phase high-performance liquid chromatography (HPLC) with fluorescence end-point detection. The calibration curve was linear over the range 5–100 ng per ml of plasma. The sensitivity for hypericin was 75 pg on column. Mean inter- and intra-assay coefficients of variation (C.V.s) over the range of the standard curve were less than 10%. The absolute recovery for hypericin averaged 72.6%.

Keywords: Hypericin

1. Introduction

Hypericin, 4,5,7,4′,5′,7′-hexahydroxy-2,2′-dimethyl-meso-naphthodianthrone, is one of the characteristic constituents of HP. Its pharmacological and therapeutic properties have been extensively reviewed [1,2]. The structure of hypericin is shown in Fig. 1.

Liebes et al. [3] reported an HPLC method for the determination of hypericin in plasma with fluorimetric end-point detection. In this method samples were chromatographed by gradient elution on a reversed-phase phenyl column, The extraction procedure was slow and each chromatographic run took about 30 min, thus making the whole procedure a lengthy one. However, the authors reported an assay sensitivity of 250 pg on column. Kerb et al. [4] reported a highly sensitive HPLC method with fluorimetric detection for the simultaneous measurement of hypericin and pseudohypericin in plasma. The quantification limit for the procedure was reported to be 0.2 μg/l when 250-μl plasma samples were used. However, this

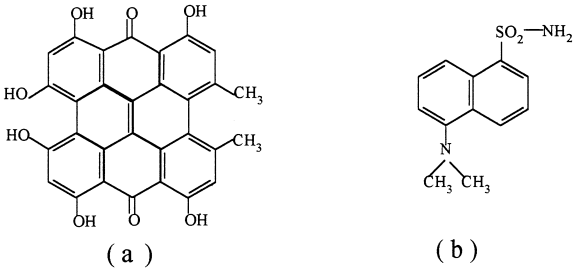

Fig. 1. Structures of (a) hypericin and the internal standard, (b) dansylamide.

*Corresponding author.

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technique used a liquid–liquid extraction procedure, which is slow, cumbersome and gives reduced analyte recovery. Another recently reported procedure utilized HPLC coupled to an electrospray mass spectrometer for determination of naphthodianthrones in plant extract [5]. This method is not generally suitable for routine measurement of hypericin in plasma because the equipment is initially costly, is expensive to maintain and requires a high degree of expertise to run.

Our aim was to set up an assay procedure that was quick, simple, cheap to run, robust, gave a high degree of selectivity and could be used in a general clinical laboratory. We describe a method which we feel satisfy these criteria. The method utilizes HPLC with fluorescence end-point detection, SPE and an internal standard, dansylamide, for monitoring both analyte recovery and detector response variations during analysis.

2. Experimental

2.1. Materials

Hypericin was donated by Lichtwer Pharma (Berlin, Germany). Dansylamide was obtained from Sigma (Poole, UK). Potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate and methanol were purchased from BDH Laboratory Supplies (Poole, UK). All reagents used for the assay were of the highest grade available. Water was deionised and glass distilled prior to use. Drug-free plasma for the preparation of calibration standard was obtained from healthy volunteers.

Stock standard solutions of hypericin and I.S. were prepared at concentrations of 100 μg/ml in methanol and when stored at 4°C were stable for at least 3 months. The assay standard was prepared freshly for each assay from stock solutions.

2.2. Chromatography

The HPLC system consisted of a Model PU-950 pump (Jasco, Tokyo, Japan), a manual Rheodyne 7125 injection valve equipped with a 50-μl loop, a 150×4.6 mm, I.D. 5-μm particle size C8 analytical column (Capital HPLC, Edinburgh, UK) and a Jasco model 821-FP spectrofluorometer (Jasco) with the excitation and emission wavelengths set to 315 and 519 nm respectively, was used for end-point detection.

The mobile phase consisted of 0.03 *M* KH_2PO_4 adjusted to pH 7.0 with 0.5 *M* K_2HPO_4 and methanol (30:70, v/v). The mobile phase was filtered and degassed prior to use. The flow-rate was 1.0 ml/min. The analytical column was kept at 60°C in a column oven (Jones Chromatography, Hengoed, UK).

Peak heights rather than peak areas in the chromatography were normally measured. Plasma concentrations of hypericin were assessed by interpolation of the standard curve.

2.3. Procedures

Blood samples were collected into tubes containing lithium heparin as anticoagulant. The plasma was prepared following centrifugation at 1500 *g* at 4°C and subsequently stored at −20°C until required for assay.

Standards for assay were prepared in duplicate in drug-free plasma and consisted of six concentration points over the range of 5–100 ng/ml and blanks. To each 0.1 ml of standard or sample was added 75 ng of the dansylamide (in methanol).

C8 sorbent columns (50 mg Isolute, Jones Chromatography) were conditioned with consecutive full column volumes (1 ml) of methanol and 0.025 *M* potassium phosphate buffer (pH 6.8). The vacuum on the manifold system (VacMaster, IST, MID Glamorgan, UK) was diverted to prevent the columns from drying out and the standards and samples were loaded on to the relevant columns. The vacuum was again applied to allow the complete passage of the materials through the column. Each column was washed with one column volume of 0.025 *M* potassium phosphate buffer (pH 6.8) and taken to dryness under vacuum. The vacuum was again diverted, the manifold needles were wiped dry and a collection tray containing 75×10 mm glass tubes was inserted into the vacuum manifold. Compounds were eluted with a single column of methanol. Eluates were evaporated to dryness under vacuum at 40°C. The residue was reconstituted in 100 μl mobile phase,

vortex mixed and a 20 μl solution was injected into the HPLC.

3. Results

Resolution and sensitivity was determined by injection of an extracted plasma standard (Fig. 2). The retention times of internal standard and hypericin were 1.9 and 3.8 min, respectively. The linearity of both extraction procedure and the detector response (determined from the peak height) was verified over the assay range (5–100 ng/ml). This was done by assaying pooled drug-free plasma (which had been previously screened for extraneous peaks) spiked with known amounts of each of the analyte. A calibration curve was calculated for the analyte using its concentration and the peak height ratio of the analyte to internal standard over the standard range.

The mean equation for the hypericin calibration curve using linear regression analysis, was $y=0.0124x+0.0015$ ($r=0.9993$, $n=6$). The inter- and intra-assay C.V.s are given in Table 1. The mean extraction recovery for hypericin QCs 10, 50 and 100 ng/ml was 72.6% (C.V.%=3.4. $n=9$). Sample extracts were stable for 1 week when stored out of light at 4°C.

The plasma profile of hypericin following oral administration of 2700 mg of HP extracts in 12 male volunteers subjects is shown in Fig. 3.

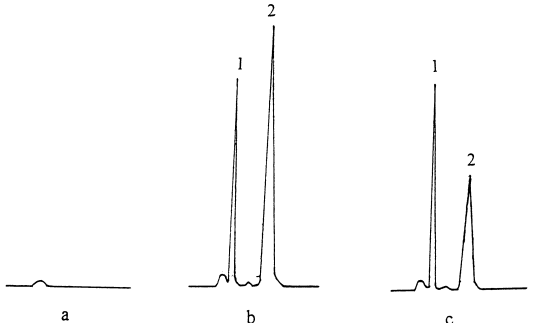

Fig. 2. Chromatogram of (1) blank drug-free plasma, (2) drug-free plasma (0.1 ml) spiked with 0.1μg/ml hypericin and 0.75 μg/ml of the internal standard. (3) Chromatogram of a sample from a volunteer following an oral dose of 2700 mg HP extracts at 240 min after dosing. Peaks 1 and 2 represent the internal standard and hypericin, respectively. The retention time for peaks 1 and 2 were 1.9 and 3.8 min, respectively, with a total run time of 4 min. The injection volume was generally 20 μl on column.

Table 1
Intra- and inter-assay precision (RSDs) and accuracy data for the determination of hypericin in plasma[a]

Actual value (ng/ml)	Observed value (ng/ml)		C.V. (%)	
	Intra	Inter	Intra	Inter
10	9.9±0.2	10.4±0.2	4.7	6.2
50	49.6±0.7	49.5±0.8	3.6	5.7
100	98.8±1.0	99.2±1.4	2.4	4.7

[a] The intra- and inter-assay data represents the mean±SEM of six and 12 observations, respectively.

4. Discussion

We described a quick, simple, and highly selective HPLC assay procedure which utilizes fluorescence detection, SPE and an internal standard for monitoring extraction recovery and detector variation. Dansylamide was chosen as the internal standard because it was known to have similar fluorescent properties to hypericin [4]. The assay is highly sensitive with a detection limit (i.e. peak height equal to three times baseline noise) of 75 pg of hypericin on column. It was established that the ratio between the analytical recovery of hypericin and that of the internal standard submitted to the same operations was constant over a wide concentration

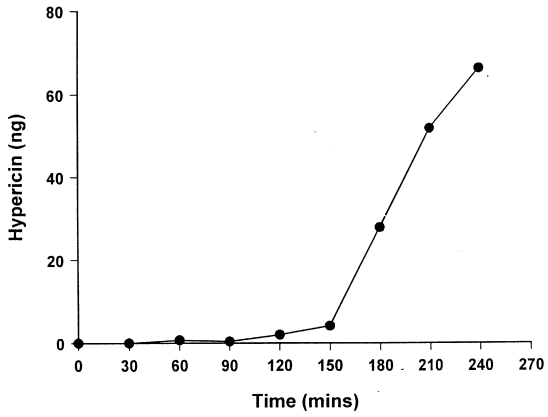

Fig. 3. Plasma concentrations of hypericin following oral dosing (2700 mg) of HP extracts in 12 male volunteers.

range. The requirements for an internal standard assay procedure were therefore satisfied.

The method demonstrates significant improvements over other published procedures. Higher recoveries, lower sample volumes (100 μl) and reduced sample preparation time have all been attained by utilisation of SPE technology. Chromatography took less than 4 min for each assay run and demonstrated good resolution of analyte without interference. The range of the standard is sufficient to incorporate the majority of expected values.

5. Conclusions

A novel, rapid, simple and highly specific HPLC method has been described for the measurement of hypericin in plasma. The assay is cheap to run and may easily be set up in a routine clinical laboratory. The method has clear advantages over previously described procedures.

References

[1] H.P. Volz, Pharmacopsychiaty 30 (Suppl.) (1997) 72–76.
[2] G. Lavie, Y. Mazur, D. Lavie, D. Meruelo, Med. Res. Rev. 15 (1995) 111.
[3] L. liebes, Y. Mazur, D. Freeman, D. Lavie, G. Lavie, N. Kudler, S. Mendoza, B. Levin, H. Hochster, D. Meruelo, Anal. Biochem. 195 (1991) 77–85.
[4] R. Kerb, J. Brockmoller, B. Staffeldt, M. Ploch, I. Roots, Antimicrob. Agents Chemother., Sept. (1996) 2087–2093.
[5] G. Piperopoulos, R. Lotz, A. Wixforth, T. Schmierer, K.-P. Zeller, J. Chromatogr. B 695 (1997) 309–316.