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Note

Determination of salicylhydroxamic acid, a trypanocidal agent, by reversed-phase high-performance liquid chromatography

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Salicylhydroxamic acid (SHAM) specifically inhibits the respiration of bloodstream forms of Trypanosoma brucei [1—3] and has therefore been used in animals as a trypanocide [3, 4]. SHAM has been determined in plasma by several methods. A biological assay utilising inhibition of trypanosome respiration by SHAM has the advantage of only measuring the therapeutic SHAM concentration [3]. However, it does not distinguish between SHAM and active metabolites. A colorimetric method has been described [5], this being a modification of an assay for hydroxamic acids developed by Bergman and Segal [6].

In this note we describe a sensitive and reproducible method of estimating SHAM in whole blood using reversed-phase high-performance liquid chromatography (HPLC). This technique is able to distinguish SHAM and salicylamide, the major metabolite of SHAM [7, 8]. It is therefore suitable for the determination of the pharmacokinetic parameters of the drug in vivo. It has also been found suitable for the estimation of SHAM in homogenates of several animal organs.

EXPERIMENTAL

Reagents

Salicylhydroxamic acid was a kind gift of Professor T. Urbanski (Technical

University, Warsaw, Poland) and phenacetin was obtained from Sigma (London) (Poole, Great Britain). All other chemicals used were of analytical grade and were supplied by BDH (Poole, Great Britain). These were used as supplied; aqueous solutions were prepared using glass-distilled water.

Extraction and chromatographic procedures

To 0.1 ml whole blood in a ground glass stoppered tube were added 30 μl internal standard solution (25 mg/ml phenacetin in methanol), 0.1 ml 0.02 *N* hydrochloric acid and 2 ml ethyl acetate. The mixture was roller mixed for 10 min and centrifuged at 1000 *g* for 5 min. A 1.5-ml aliquot of the supernatant organic phase was transferred to a conical tube and evaporated to dryness under a stream of air, in a water bath at 55°C. The residue was dissolved in 0.2 ml methanol and a 20-μl aliquot was injected onto the column.

SHAM standard samples were made up by adding aliquots of a 1 mg/ml SHAM solution (in methanol) to drug-free blood.

High-performance liquid chromatography

The chromatograph was a Pye-Unicam LC-XPS pump equipped with a 15 × 0.5 cm I.D. stainless steel column packed with Magnusphere (5 μm) C_{22} reversed-phase packing obtained from Magnus Scientific (Sandbach, Great Britain) and fitted with a Model 701 Rheodyne injection valve and a 20-μl injector loop. The apparatus was operated at ambient temperature. The eluting solvent was a degassed mixture of 0.043 *M* ammonium dihydrogen phosphate–methanol (70:30, v/v). The pH of this mixture was adjusted to 2.0 by the addition of approximately 5 ml orthophosphoric acid per litre of 0.043 *M* ammonium dihydrogen phosphate. The instrument was operated at a constant flow-rate of 1.2 ml/min and the absorption of eluent was monitored at 300 nm using a Pye-Unicam LC3 UV variable-wavelength absorbance detector.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 1. The retention volumes for SHAM and phenacetin were 3.5 and 10.2 ml, respectively. A chromatogram of a blank blood sample is shown for comparison. No interfering peaks due to endogenous substances were present in the blank sample.

The linearity of the assay was determined by extracting five standard solutions in blood containing 0, 10, 20, 50 and 75 μg/ml SHAM. The correlation coefficient, *r*, was 0.991. Reproducibility was assessed at 1, 10, 20 and 50 μg/ml using six samples at each concentration. The results are shown in Table I.

The minimum level of detection for SHAM using this assay was 0.1 μg/ml from a 0.1-ml blood sample.

The accuracy of the assay was determined by analysis of twelve blood samples spiked at unknown concentrations. The mean percentage error between the known spiked concentration and that estimated was 8.9%.

The recovery of SHAM from the extraction procedure was 89% at 5 μg/ml, 83% at 30 μg/ml and 86% at 100 μg/ml. The principal metabolite of SHAM

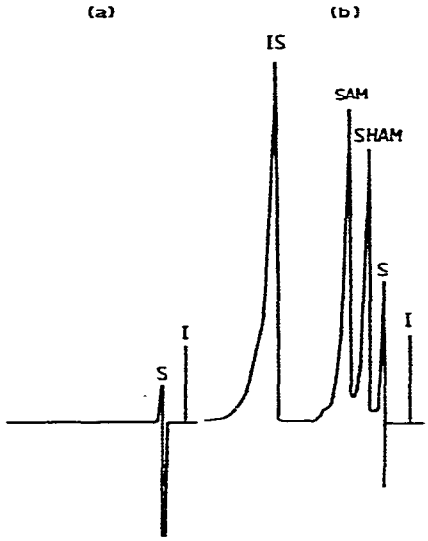

Fig. 1. Representative chromatograms for (a) blood containing no drug; (b) sample of a kidney homogenate taken following administration of 200 mg/kg SHAM to a mouse. Peaks: I = injection; S = solvent; SHAM = salicylhydroxamic acid; SAM = salicylamide; IS = internal standard. The concentrations of SHAM and SAM in this sample were 18 μg/g and 16.2 μg/g, respectively.

TABLE I

COEFFICIENTS OF VARIATION FOR SHAM DETERMINATIONS
$n = 6$ at each concentration.

SHAM concentration (μg/ml)	S.D.	C.V. (%)
1	0.065	6.2
10	0.045	4.0
20	0.126	6.6
50	0.200	4.0

is salicylamide [7, 8] and can be extracted in the same way as SHAM and estimated using this HPLC system. It has a retention volume of 7.4 ml and can therefore be separated from both SHAM and the internal standard.

Linearity of the method for salicylamide was confirmed by extracting samples containing 0, 40, 80 and 100 μg/ml. The correlation coefficient, r, was 0.999.

This assay provides a rapid and reproducible method for determining SHAM concentrations in small volumes of whole blood, plasma or tissue homogenates. It requires much smaller volumes of blood and is more accurate than methods for SHAM estimation described hitherto [3, 5]. The ability of this HPLC system to distinguish SHAM from salicylamide and other hydroxamic acids makes it suitable for determination of the pharmacokinetics of the parent compound which is known to be an active trypanocide [1–3]. The sensitiv-

ity is sufficient to measure SHAM in very small blood samples allowing for its use in the study of SHAM pharmacokinetics in the rodent models of the trypanosome infections of man and larger animals which are so widely used in the laboratory research into these infections (Fig. 2).

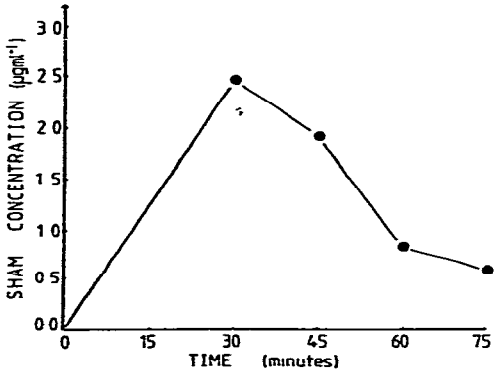

Fig. 2. Mean blood SHAM concentrations following oral administration of 200 mg/kg SHAM to five mice (mean elimination half-life of SHAM = 21 min).

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AB and Wv'tH held MRC student awards.

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REFERENCES

1 D.A. Evans and R.C. Brown, J. Protozoology, 20 (1973) 157.
2 D.A. Evans and R.C. Brown, Trans. Roy. Soc. Trop. Med. Hyg., 67 (1973) 258.
3 F.R. Oppendoes, P.N. Aarsen, C. van der Meer and P. Borst, Exp. Parasitol., 40 (1976) 198.
4 A.B. Clarkson and F.H. Brohn, Science, 194 (1976) 204.
5 C. van der Meer, J.A.M. Versluij-Broers and F.R. Oppendoes, Exp. Parasitol., 48 (1979) 126.
6 F. Bergman and R. Segal, Biochem. J., 62 (1956) 542.
7 J. Lowenthal, Nature (London), 174 (1954) 36.
8 W.M. McIsaac and R.T. Williams, Biochem. J., 66 (1957) 369.