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# DETERMINATION OF INDIVIDUAL HYDROXYETHYL RUTOSIDES IN VARIOUS ANIMAL BODY FLUIDS BY THIN-LAYER CHROMATOGRAPHY AND SCANNING DENSITOMETRY

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## SUMMARY

A method is described for the accurate determination in plasma of the $\beta$-hydroxyethylrutosides by measurement of the fluorescence of their borocitrate complexes by scanning densitometry following separation by thin-layer chromatography. A modification is also described for estimation of individual hydroxyethylrutosides and their glucuronide conjugates in samples of bile and urine.

## INTRODUCTION

Although the hydroxyethylrutoside preparation Venoruton® (Zyma, Nyon. Switzerland) has been widely used in the treatment of a number of vascular disorders[1–5], analytical methods suitable for the monitoring of hydroxyethylrutoside levels in replicated samples of blood and urine during larger scale clinical studies have not yet been reported.

A method for the accurate determination of each of the major components of Venoruton [*i.e.* 3′,4′,5,7-tetra-O-($\beta$-hydroxyethyl)rutoside,3′,4′,7-tri-O-($\beta$-hydroxyethyl)rutoside and 4′,7-di-O-($\beta$-hydroxyethyl)rutoside] in plasma following intravenous administration has now been developed. Recent studies[6,7] quantified the excretion of hydroxyethylrutosides in bile and urine following intravenous administration of single hydroxyethylrutosides by UV spectrophotometry, since absorption between 340 and 360 nm (Band 1) of the hydroxyethylrutosides obeys the Lambert-Beer law over the range 10–50 $\mu$g (ref. 2). Since the UV spectra of the constituent hydroxyethylrutosides of Venoruton are similar[8,9] it is not possible to quantify specific ruto-

sides in solution without prior separation. Such a separation can be achieved using the method of Johnson et al.[10] as modified by Hackett and Griffiths[11], but this method is not suitable for the rapid handling of large numbers of samples.

A thin-layer chromatographic (TLC) separation of the hydroxyethylrutosides and their metabolites in perfusion media with subsequent quantification of the rutosides by measurement of their fluorescence following spraying with methanolic aluminium chloride has been reported[12,13], but the spraying of aluminium chloride onto plates has been found in this laboratory to give rise to variability in the densitometric results obtained.

A rapid, sensitive and reproducible method for the determination of hydroxyethylrutosides in plasma and tissue fluids by TLC followed by *in situ* fluorescence densitometry is now described.

EXPERIMENTAL

*Materials*

Venoruton, 3',4',7-tri-O-($\beta$-hydroxyethyl)rutoside (tri-HR), 3',4',5,7-tetra-O-($\beta$-hydroxyethyl)rutoside (tetra-HR), 4',7-di-O-($\beta$-hydroxyethyl)rutoside (di-HR) and 7-mono-O-($\beta$-hydroxyethyl)rutoside (mono-HR) were supplied by Zyma, Nyon, Switzerland. Silica gel G Type 60 (Merck, Darmstadt, G.F.R.) was used in the preparation of thin-layer plates. Other reagents and solvents (analytical reagent grade) were obtained from commercial sources. Blood plasma was obtained by centrifugation of heparinized rabbits blood. Dog plasma was obtained similarly. Urine was collected from rats and dogs and filtered before use. Bile was obtained from rats by implantation of a plastic cannula into the common bile duct with animal under nembutal anaesthesia[1].

*Preparation of thin-layer plates*

300-$\mu$m-thick layers were prepared by shaking 30 g of silica gel G Type 60 with either 60 ml of distilled de-ionized water or with 60 ml of an aqueous solution containing 3 % boric- and 1 % citric acid and spreading the slurry on to the glass plates with a Shandon Unoplan spreader. These quantities were sufficient to make five 20 × 20 cm thin-layer plates. Plates were dried in air for 30 min and then heated in an oven at 130° for 5 min, allowed to cool and stored in a desiccating cabinet.

*Thin-layer chromatography*

Samples of blood plasma containing hydroxyethylrutosides (HRs) were deproteinated by addition of an equal volume of acetone and centrifuged. The supernatant was taken to dryness and the residue redissolved in a suitable volume of methanol for chromatography.

Standard solutions of each HR in urine or bile were prepared such that suitable microgram ranges of specific HRs could be applied to the plates in the same volume of the appropriate biological fluid. Equal volumes of fluids containing unknown levels of the various HRs were applied to the plates alongside the calibration ranges, using a Hamilton Ziptrol microlitre delivery system. The spots were dried in a stream of warm air. Plates were developed in either ethyl acetate–formic acid–water (70:15:15, v/v/v) (solvent system I) or ethyl acetate–formic acid–water (100:25:32,

v/v/v) (solvent system II), and were dried in a stream of air for 2 h to remove all traces of solvents before scanning.

## Scanning densitometry

All measurements were made using a Vitatron TLD 100 flying spot densitometer with appropriate filters and settings (see Table I). Thin-layer plates (20 × 20 cm) bearing the calibration standards and test solutions were scanned horizontally across the plate at the $R_F$ of the component to be measured, or vertically from the origin to the solvent front to detect the various rutosides present in the samples. Measurements were made and recorded on an integrating chart recorder linked to the densitometer.

TABLE I

OPTIMAL CONDITIONS FOR THE DETERMINATION OF HR-COMPLEXES IN THE VITATRON TLD 100 FLYING SPOT DENSITOMETER

Plates: borate–citrate impregnated silica gel plates; solvent system: either solvent I or solvent II.

| HRs | Mode | Diaphragm size | Prediaphragm | Filters | | |
|-----|------|----------------|--------------|---------|---|---|
| | | | | Lamp | Activation | Emission |
| Mono-HR | LinII─ | 1.0 mm | Removed | Hg | UVB | IFL 525 |
| Di-HR | LinII─ | 0.5 mm | Removed | Hg | UVB | IFL 525 |
| Tri-HR | LinII─ | 0.5 mm | Removed | Hg | UVB | IFL 525 |
| Tetra-HR | LinII─ | 0.25 mm | Removed | Hg | UVB | IFL 456 |

## Determination of conjugated hydroxyethylrutosides

Glucuronide conjugates of specific hydroxyethylrutosides may be determined by dissolving the deproteinated, freeze-dried plasma samples in 0.1 $M$ KH$_2$PO$_4$–Na$_2$HPO$_4$ buffer (pH 6.8) and measuring the specific HR by scanning densitometry before and after incubation of the buffered plasma samples with $\beta$-glucuronidase (Bacterial type 1, Sigma, Kingston-on-Thames, Great Britain) for 24 h at 37° (ref. 7).

## RESULTS AND DISCUSSION

### Determinations of Venoruton constituents in rabbit plasma

When known quantities of tetra-HR (which, when irradiated with UV light, has a strong native blue fluorescence) were applied in methanol to either silica layers or silica layers impregnated with boric and citric acids in volumes up to and including 25 $\mu$l, the relationship between light emitted at 456 nm and the amount of tetra-HR was shown to be linear over the ranges used (viz. 0.1–0.5 $\mu$g and 1–5 $\mu$g) after development in either solvent system I or II (Fig. 1). Similar experiments with known amounts of tri-HR and di-HR developed on silica layers impregnated with boric and citric acids showed a linear relationship between the amount of rutoside and light emitted at 525 nm over the ranges used (viz. 0.1–0.5 $\mu$g and 1–5 $\mu$g) (Fig. 1). It is important to note that tri- and di-HR do not in the absence of borocitrate produce measurable fluorescence on silica gel plates.

Chromatography of Venoruton and varying mixtures of di-, tri- and tetra-HRs extracted from deproteinated rabbit plasma under the conditions described
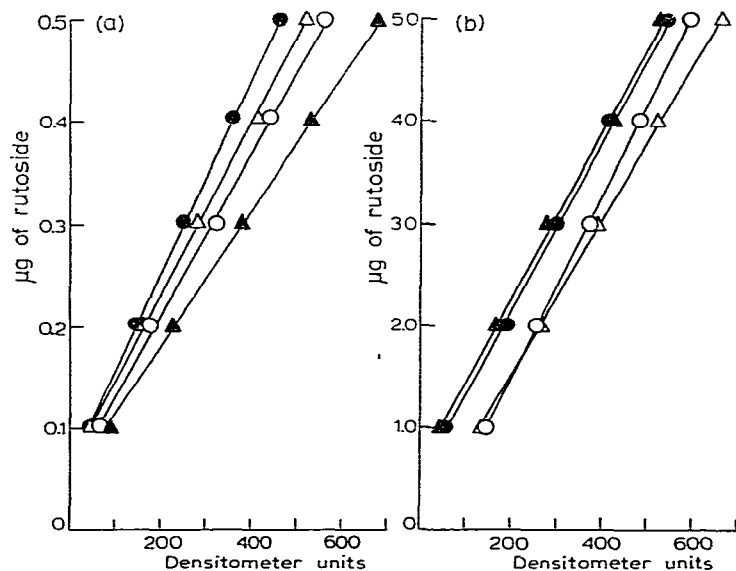
Fig. 1. Calibration curves for hydroxyethylrutosides under the conditions shown in Table I. Range: (a) 0.1–0.5 μg; (b) 1–5 μg. ▲, Mono-HR; △, di-HR; ●, tri-HR; ○, tetra-HR.

showed that the individual components were well resolved into discrete spots (Table II and Fig. 2). Methanolic extracts of dried samples of deproteinated plasma from rabbits taken at various times after a large intravenous infusion of Venoruton were equally well separated (Fig. 2) and by using a suitable dilution of deproteinated plasma extract and the appropriate calibration standards, HR concentrations from 1 to 2500 μg/ml of plasma were measured. The solvent systems used also gave good separation between the unchanged HRs and their respective glucuronide conjugates which are their major tissue metabolites in a variety of species[7,14], and between these two groups of compounds and endogenous fluorescent plasma components which were located primarily at the solvent front and origins of TLC plates. The HR-glucuronides were quantified following their enzymic hydrolysis to the corresponding HRs when the increase in each HR was determined (see Experimental).

## TABLE II

### $R_F$ VALUES AND FLUORESCENCE CHARACTERISTICS OF HYDROXYETHYLRUTO-SIDES ON UNTREATED SILICA GEL PLATES AND ON BORATE–CITRATE IMPREGNATED SILICA GEL PLATES

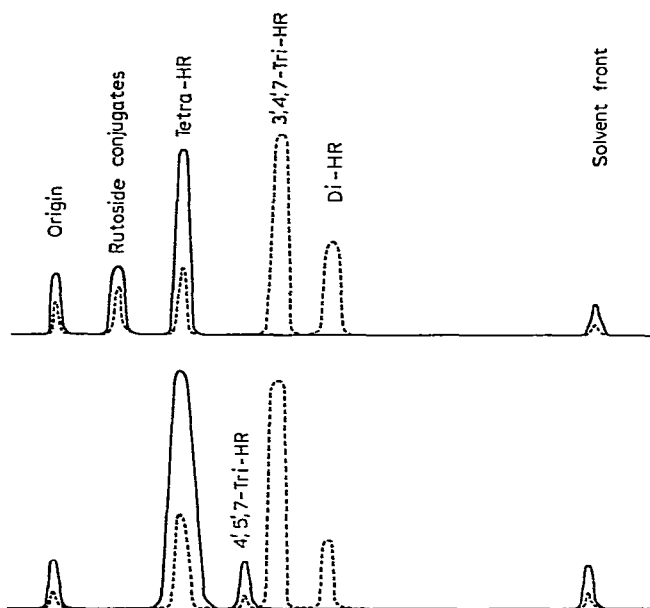| HRs | $R_F$ values | | | | Appearance under UV | |
|---|---|---|---|---|---|---|
| | Ordinary plates | | Borate–citrate plates | | Ordinary plates | Borate–citrate plates |
| | Solvent I | Solvent II | Solvent I | Solvent II | | |
| Mono-HR | 0.52 | 0.64 | 0.44 | 0.56 | not visible | yellow-green |
| Di-HR | 0.44 | 0.50 | 0.34 | 0.51 | not visible | yellow-green |
| Tri-HR | 0.33 | 0.42 | 0.25 | 0.41 | not visible | yellow-green |
| Tetra-HR | 0.17 | 0.25 | 0.12 | 0.23 | blue | blue |

Fig. 2. Densitometer scans of Venoruton (lower scan) and of deproteinated rabbit plasma following venous infusion of Venoruton (upper scan). Scans were obtained at 456 nm (complete lines) and 525 nm (broken lines).

*The determination of 7-mono-O-(β-hydroxyethyl)rutoside in plasma*

The compound 7-mono-O-(β-hydroxyethyl)rutoside, which is not a constituent of Venoruton but is currently undergoing pharmacological[5] and metabolic investigation[7], may also be determined by scanning densitometry.

Methanolic extracts of deproteinated dog plasma were prepared by the same method as those for the rabbit. Like tri-HR and di-HR, mono-HR has no native fluorescence under UV light, but does form a complex with boric acid and citric acid which fluoresces yellow-green under UV light. The major metabolite of mono-HR in body fluids, a glucuronide conjugate[7], exhibits similar properties. The chromatographic systems described gave clear separations of mono-HR from endogenous fluorescent plasma components of deproteinated extracts, and when deproteinated plasma extracts from a dog given an intravenous injection of mono-HR were examined in this way, clear separation of mono-HR and its glucuronide were observed.

The relationship between the light emitted at 525 nm following UV irradiation and the quantity of mono-HR was found to be linear over the ranges 0.1–0.5 $\mu$g and 1–5 $\mu$g (Fig. 1). Unchanged mono-HR in dog plasma samples can thus be estimated by scanning densitometry of the appropriate standards and experimental deproteinated plasma samples.

*The determination of hydroxyethylrutosides in urine and bile samples*

Good separations were also obtained using the conditions described when the HRs were contained in 20 $\mu$l of the urine of rats or dogs or in 20 $\mu$l of the bile of rats. Linear relationships between the amounts of HR and light emission at 525 nm for

mono-, di- and tri-HRs and 456 nm for tetra-HR were noted over the ranges described for plasma samples when run on boric acid–citric acid impregnated silica layers.

Following the determination by TLC and scanning densitometry under the described conditions of known amounts of each of the hydroxyethylrutosides following addition of the pure compounds to normal plasma and urine, the standard deviations shown in Table III were obtained.

The quantification of plasma and urine samples containing known amounts of hydroxyethylrutosides under the conditions described indicated that recovery of hydroxyethylrutosides was 100% from urine and 96.6 $\pm$ 3.82% from plasma samples.

TABLE III

STANDARD DEVIATIONS (%) FOR THE MEASUREMENT BY TLC-SCANNING DENSITOMETRY OF HYDROXYETHYLRUTOSIDES IN STANDARD SOLUTION IN PLASMA AND IN URINE

Results are quoted for four determinations on each biological fluid containing 20 mg of the specific hydroxyethylrutoside/100 ml under the experimental conditions described in the text.

|  | Mono-HR | Di-HR | Tri-HR | Tetra-HR |
|---|---|---|---|---|
| Urine | $\pm$ 2.07 | $\pm$ 2.31 | $\pm$ 2.23 | $\pm$ 1.96 |
| Plasma | $\pm$ 2.35 | $\pm$ 2.41 | $\pm$ 2.41 | $\pm$ 2.05 |

CONCLUSIONS

Hydroxyethylrutosides present in biological fluids may be satisfactorily separated and quantified by a TLC method utilizing the known ability of flavonols possessing a free 5-hydroxyl group to chelate with the borocitrate reagent to give yellow coloured complexes[15] which fluoresce intensively at microgram levels under the conditions employed. Although deproteinization of plasma samples was found to be necessary for optimal results, direct application of samples of bile and urine containing HRs was found to give satisfactory separation and quantification of the HRs and their glucuronide conjugates.

Tetra-HR, although possessing a 5-hydroxyethyl substituent, may also be determined fluorimetrically on these plates, for although substitution in this position prevents chelation with the borocitrate reagent, 5-substitution of HRs gives rise to blue fluorescence[8,9] which may also be determined at the appropriate wavelength.

Since a large number of other flavonol glycosides are known to chelate with the borocitrate reagent[15] and to give rise to discrete spots in the solvent systems employed[14], the method described may prove of general utility in the determination of naturally occurring flavonol glycosides.

ACKNOWLEDGEMENTS

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