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Note

Simultaneous assay of antipyrine and its major metabolites in urine using high-performance liquid chromatography and on-line solid phase sample clean-up

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The therapeutic blood levels of numerous drugs can be influenced by inter-individual variations in hepatic metabolism. Antipyrine elimination half-life has often been used for investigating hepatic oxidation [1, 2].

Phase I metabolism of antipyrine (AP) yields three major oxidative products, norantipyrine (NORA), 4-hydroxyantipyrine (OHA) and 3-hydroxymethyl-antipyrine (HMA), which, in rats, are produced by three different hepatic P-450 cytochromes [3—6]. In man 4,4′-dihydroxyantipyrine (3—6% oral dose) as well as 4′-hydroxyantipyrine (2—4% oral dose) are also formed, but their levels are negligible [7].

These metabolites undergo conjugation to glucuronide or sulphate conjugates [7, 8]. Up to 80% of the oral dose is excreted as the three major metabolites in both the free and conjugated states [9, 10]. In man, OHA and NORA are almost completely glucuronidated, whilst HMA is variably conjugated or excreted as the free metabolite [7].

Because the major metabolic reactions proceed at different rates, the rate of antipyrine elimination from plasma or saliva alone cannot provide kinetic information concerning each of these separate reactions. To do this, the rate of appearance of each major metabolite needs to be determined.

Several methods for the assay of antipyrine alone in plasma and saliva have been published.

Initially the drug was determined spectrophotometrically [11, 12] but later gas chromatographic (GC) methods with flame-ionization detection (FID) and nitrogen—phosphorus detection (NPD) [13—21] were developed.

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Whilst these methods are sufficiently selective and sensitive for antipyrine in plasma, serum or saliva, only two methods, described in refs. 19 and 20, are applicable to antipyrine metabolites in urine, and then only for OHA and NORA but not for the more polar HMA.

Thin-layer chromatography has been used for the assay of 4,4'-dihydroxy-antipyrine [22], OHA and HMA [4, 22] and the conjugated metabolites [7], but involved either the use of labelled antipyrine [4, 7] or extensive sample preparation including hydrolysis for 60 min at pH 1 [22] in which NORA is unstable [28].

Numerous high-performance liquid chromatographic (HPLC) methods for the assay of antipyrine in serum, plasma and saliva [23–27] and its metabolites in urine [3, 9, 20, 28–30] have been published. The results obtained with these methods have been very variable, mainly due to the instability of the metabolites at pH < 2 or pH > 6 [28], oxidation of NORA and OHA, and the different extraction systems used.

Danhof et al. [9] used sodium metabisulphite to minimize oxidation of OHA whilst Teunissen et al. [28] used it to prevent oxidation of NORA and OHA, but then subjected the samples to an alkaline extraction procedure at a pH where both NORA and OHA are unstable.

In this paper, a reversed-phase HPLC (RP-HPLC) method is presented for the sample clean-up and simultaneous assay of antipyrine and its metabolites in urine. On-line solid phase clean-up of the directly injected deconjugated urine on a standard commercial high-performance liquid chromatograph with a loop injector valve, and with no further column switching valves, is described.

EXPERIMENTAL

Reagents

All solvents used were spectroscopic grade from Burdick & Jackson (Muskegon, MI, U.S.A.) and all water was purified by the Milli-Q system (Millipore). The other reagents were analytical reagent grade. Glusulase was obtained from DuPont Pharmaceuticals (Wilmington, DE, U.S.A.). A sample buffer (pH 4.7) of 0.5 *M* ammonium dihydrogen phosphate with 80 g/l sodium metabisulphite as antioxidant was used to dilute samples and standards.

Standards

Antipyrine was purchased from Sigma (St. Louis, MO, U.S.A.). Norantipyrine and 4-hydroxyantipyrine were obtained from Aldrich (Gillingham, U.K.) and 3-hydroxymethylantipyrine was generously donated by Professor D.D. Breimer (University of Leiden, Leiden, The Netherlands). Because of the instability of NORA, no stock solutions of the standards were used [28]. All standard solutions were prepared in sample buffer in concentrations of 1000, 500, 200, 100 and 50 μg/ml antipyrine and each metabolite daily. When the standard samples for injection are prepared as described below, the effective concentration is half that of the standard solution used.

Internal standard

Phenacetin was found to be satisfactory as an internal standard in the elution

and detection conditions used. Since it is stable in aqueous solution, a stock solution of 1 mg/ml in sample buffer was made and stored for up to one month at 4°C.

Sample collection

Antipyrine (600 mg) was administered orally to each fasting volunteer and urine collected for the periods 0—3, 3—8, 8—24, 24—32 and 32—48 h. Sodium metabisulphite (ca. 4 mg/ml) was added as an antioxidant to all collection bottles and 25-ml samples from these were then frozen in liquid nitrogen and stored at −18°C for no more than ten days prior to assay.

Sample preparation

A 200-μl sample of urine was added to 200 μl sample buffer containing sodium metabisulphite and 50 μl internal standard (1 mg/ml) in a borosilicate tube and vortexed for 5 s. To this, 20 μl Glusulase (ca. 2300 U β-glucuronidase and 1100 U sulphatase) were added and the contents gently mixed by inverting the stoppered tube twice. The mixture was then incubated at 37 ± 0.4°C for exactly 2.5 h. The samples were then rapidly frozen in liquid nitrogen and stored at −18°C for up to one week for later assay. This permitted batch preparation of large numbers of samples which were individually defrosted immediately before asay and mixed with a further 100 μl of sample buffer. A volume of 25 μl of the resulting mixture was injected into the chromatograph.

Preparation of standards

Blank urine was incubated in the same manner as the urine samples. Just prior to injection 100 μl of a solution of standards were added to the incubated blank urine and 25 μl of this mixture injected into the chromatograph.

Chromatography

HPLC separation was performed on a Spectra-Physics 8100 liquid chromatograph with a Valco autoinjector loop valve. The sample loop was replaced by a self-packed 20 mm × 4.6 mm I.D. pre-column filled with Supelco 30 μm LC-18

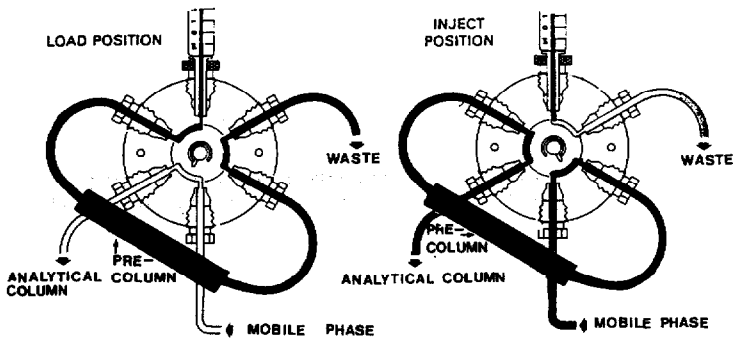

Fig. 1. Clean-up column and injector valve assembly. The reversed-phase clean-up column replaces the loop in the Valco loop injector valve. Load position: the sample is loaded onto the clean-up column and the very polar urine components are flushed to waste. Inject position: the analytes are flushed, under high pressure, out of the clean-up column and onto the analytical column by mobile phase.

pellicular packing (Fig. 1). The analytical column was a 250 mm × 4.6 mm I.D. Spherisorb S5 ODS2. Isocratic binary elution was performed with a mobile phase of acetonitrile—0.05 *M* phosphate buffer (pH 5.7) containing 1 ml triethylamine per litre (10:90). The flow-rate was 2 ml/min and the column temperature was 35°C. UV detection was at 244 nm and the detector output was recorded simultaneously on a Perkin-Elmer strip chart recorder and a Spectra-Physics SP4200 integrator.

Analytical procedure

With the injector valve in the load position, a 25-μl sample of deconjugated urine was injected manually onto the pre-column with a gas-tight syringe. This was immediately followed by the slow injection of 350 μl water over 15 s to flush the very polar components to waste. The valve was then turned to the inject position to wash the required components onto the analytical column. After 3 min the injector valve was returned to the load position, so essentially heart-cutting the required components with the pre-column. The pre-column was regenerated by slowly injecting 2 ml acetonitrile, to flush late-eluting components to waste, followed by 2 ml water, leaving the pre-column ready for the following sample to be loaded and flushed just prior to the next injection time.

The body fluid concentrations of antipyrine and its metabolites were estimated on the basis of peak-height ratios from the standards calibration curves.

RESULTS AND DISCUSSION

Sample preparation and clean-up are the two aspects of biological assay which most distinguish it from formulations assay. Interfering substances which could influence the accuracy of the determination or damage the analytical column must be removed or converted prior to assay. Acidic or basic analytes can be extracted from the biological matrix by aqueous—organic partitioning steps at the appropriate pH. However, polar compounds are notoriously difficult to quantitatively separate from a biological matrix.

To assay the antipyrine metabolites, a hydrolysis step was included at the sample preparation stage to yield the less polar phase I metabolites which are more easily separable on a reversed-phase column. Potentially this can be achieved by acid or enzymatic hydrolysis, but because of the sensitivity of NORA to a low pH, enzymatic hydrolysis was used.

Two enzymes, β-glucuronidase from limpets and Glusulase, have variously been used for this deconjugation in published analytical methods [3, 4, 9, 19, 20, 28]. β-Glucuronidase from limpets is mixed with sulphatase and has a β-glucuronidase/sulphatase activity of approximately 65:1. Glusulase from *Helix pomatia* is again a mixed enzyme extract and has a β-glucuronidase/sulphatase activity of approximately 2:1.

About 10% of the antipyrine metabolites are excreted as sulphates [7], thus Glusulase gives the required range of sulphatase activity needed, without excess β-glucuronidase activity as is found in the limpet extract. This decrease in enzyme protein required for deconjugation reduces the amount of protein-bound OHA (0.25 μg/mg of protein) [31] in the prepared sample.

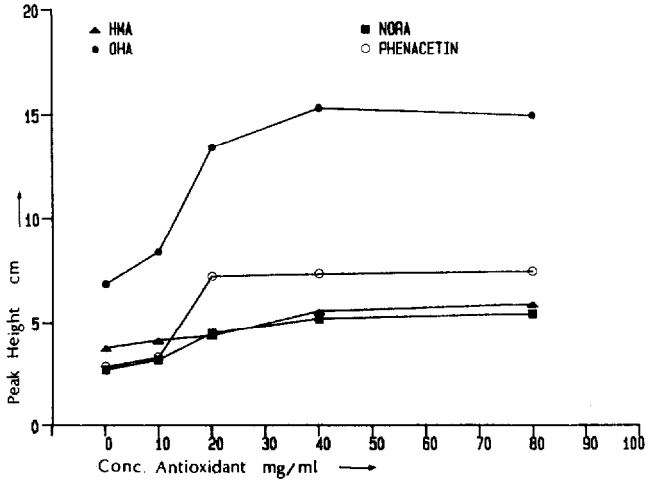

Fig. 2. Effect of anti-oxidant, sodium metabisulphite, on quantities of each metabolite found in the same urine sample after incubation with Glusulase.

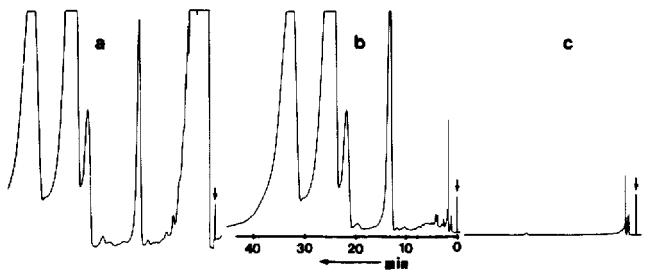

Fig. 3. Effects of heart-cutting with the pre-column on the chromatograms of blank urine from one of the volunteers. Flow-rate: 3 ml/min. (a) Deconjugated urine (25 μl) loaded onto the pre-column and injected without heart-cutting (0.16 a.u.f.s.). (b) Same urine sample (25 μl) injected after flushing sample with 350 μl water on the pre-column (0.08 a.u.f.s.). (c) Same urine sample (25 μl) after full heart-cutting by flushing sample with 350 μl water on the pre-column, injecting to allow analytes onto the analytical column, and then returning valve and pre-column to load position and flushing off the highly retained components of the urine with acetonitrile (0.32 a.u.f.s.).

A volume of 20-μl of Glusulase added to 200 μl urine in sample buffer containing 80 mg/ml sodium metabisulphite was found to give a maximal yield of metabolites from the 3–8 h urine samples after incubation at 37°C for 2.5 h (Fig. 2).

Direct injection of the incubated urine onto the analytical column yielded chromatograms, where the HMA and to some extent the NORA peaks were lost in the tail of polar urine components (Fig. 3), which remained persistently troublesome in the various elution conditions investigated. Since it was considered that an alkaline extraction should preferably be avoided, alternative methods for the separation of the analytes were investigated.

Interest has recently developed in disposable, short, pre-packed solid phase columns for the clean-up of biological samples [32—34] but, whilst the method is effective, the columns are rather expensive and designed for once-only use.

Direct injection methods for plasma and urine have also been developed [35,

36] using a clean-up column prior to the analytical column and controlling flow through the columns by either one or two additional switching valves. The cost of installing these valves and their controller is high, but the extensive applicability of on-line sample clean-up is very attractive. Thus the author investigated the use of a pre-column inserted into the loop position of a standard Valco loop injector valve and used gas-tight syringes to supply the flushing solvents required.

Self-packed 5-μm ODS pre-columns gave too high a back-pressure for the gas-tight syringe so a 30-μm ODS packing which gave a back-pressure suitable for manual flushing was used. The elution times of the analytes on the pre-column with various solvents were investigated in the same way as for independent solid phase clean-up. A 350-μl volume of water left the analytes on the pre-column, but removed much of the interfering polar components. A total of 6 ml of mobile phase (3 min at 2 ml/min) was sufficient to elute the analytes off the pre-column and onto the analytical column and 2 ml acetonitrile removed late-eluting components. The column could be regenerated with 2 ml water. It was found that the pre-column had to be repacked after 60 samples to maintain peak shape. No problems were encountered with the analytical column which has now been used for separating antipyrine or its metabolites in more than 800 urine, serum and saliva samples.

Using the above techniques, excellent separation and detectability of the analytes in urine could be achieved, as can be seen in the chromatograms (Fig. 4).

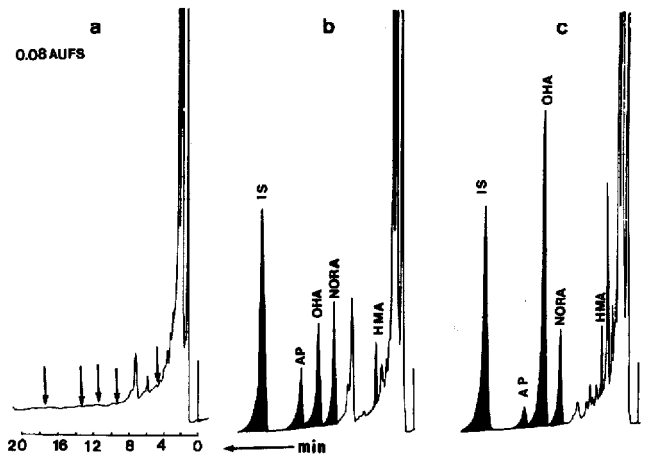

Fig. 4. Chromatograms obtained using the pre-column clean-up. Column: 250 mm × 4.6 mm I.D. Spherisorb ODS. Flow-rate: 2 ml/min. (a) Blank urine, with elution times of the analytes and the internal standard (IS) arrowed. (b) Standards (25 μg/ml) in blank urine. (c) Volunteer, 24—32 h sample after a dose of 600 mg antipyrine.

The standards response curves were linear over the range tested (10—1000 μg/ml) and the inter-sample percentage standard errors were 0.98, 0.79 and 0.88% for HMA, NORA and OHA, respectively, over five samples. The on-column detectability was 100, 50, 50 and 100 ng for HMA, NORA, OHA and AP, respectively.

Thus this rapid method of sample clean-up both prevents breakdown of the metabolites during sample preparation and eliminates interfering urinary components, allowing a highly reproducible assay of antipyrine and its major metabolites.

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